DOCTORAL THESIS

Impact of Global Warming in Holarctic and Neotropical communities of amphibians

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Impact of Global Warming in Holarctic and Neotropical communities of amphibians

Memoria presentada por Marco Jacinto Katzenberger Baptista Novo para optar al grado de Doctor en Biologia Molecular y Biomedicina: línea de Fisiología Animal, por la Universidad de Sevilla.

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“Recognize that the very molecules that make up your body, the atoms that construct the molecules, are traceable to the crucibles that were once the centres of high mass stars that exploded their chemically rich guts into the galaxy, enriching pristine gas clouds with the chemistry of life. So that we are all connected to each other biologically, to the earth chemically and to the rest of the universe atomically. That's kinda cool! That makes me smile and I actually feel quite large at the end of that. It's not that we are better than the universe, we are part of the universe. We are in the universe and the universe is in us. We are not figuratively, but literally stardust.”

— Neil deGrasse Tyson

Astrophysicist and Director of the Hayden Planetarium

To my family,

present, past and future

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GENERAL INTRODUCTION

Species and climate change

Climate change has been a part of Earth's history since its beginnings and in the past there have been periods of heating and cooling of Earth's atmosphere (Zachos et al. 2001). During the 20th century, Earth's mean temperature has already increased 0.6°C (IPCC 2013). Current predictions by the IPCC (2013) for the 21st century estimate that mean temperature will increase up to 3°C and be accompanied by an increase of extreme climatic events (Schär et al. 2004, Diffenbaugh and Ashfaq 2010) and alteration of global precipitation patterns. These climate changes have already caused changes in the phenology and geographic distribution of several species, including endothermic and ectothermic vertebrates, (Walther et al. 2002, Parmesan and Yohe 2003, Genner et al. 2004, Nussey et al. 2005, Pearce-Higgins et al. 2005, Parmesan 2006, Lenoir et al. 2008, le Roux and McGeoch 2008, Chen et al. 2009, Steltzer and Post 2009) and concern has been raised over further erosion of diversity by an extinction process which may already be underway (Sinervo et al. 2010). Predicting the impacts of climate changes in the assemblage of species and biomes is currently one of the big challenges of the scientific community (Schwenk et al. 2009).

As temperature affects virtually all physiological processes, by determining rates of chemical reactions (Hochachka and Somero 2002) and many ecological interactions (Dunson and Travis 1991), forecasting biological responses to global warming requires understanding how a species' physiology varies through space and time (Kearney and Porter 2009, Helmuth et al. 2010) and assessing how changes in physiological function due to increasing temperature may interact with phenotypic changes caused by other types of environmental variation (Chown and Terblanche 2007, Pörtner and Farrell 2008, Hoffmann 2010, Chown et al. 2010). Furthermore, determining how close organisms are to their thermal limits in nature and knowing how organisms are able to adjust or acclimatize their thermal sensitivity (Stillman 2003, Gilman et al. 2006) will help identify which species are more susceptible to global warming. Species with low tolerance to warming, limited acclimation ability, reduced dispersal, and/or that are unable to behaviourally compensate environmental changes are less likely to be able to avoid or adjust to new challenging conditions and therefore can be more vulnerable to rapid environmental changes. In addition, response to selection on thermal sensitivity is expected to be relatively fast in species that have short generation times, pronounced heritable variation in thermal sensitivity, large population size, limited inbreeding and thermally specialized physiologies (Huey and Kingsolver 1993, Kearney et al. 2009a, Kingsolver 2009, Chevin et al. 2010, Huey et al. 2012). Therefore, it is also important to consider the species' evolutionary potential and thus, if they are genetically capable of keeping pace with shifting climates or whether they will increasingly lag behind and eventually go extinct (Huey et al. 2012).

Most of the animal terrestrial biodiversity is comprised by ectotherms and, given that their physiology, development and behaviour are strongly affected by temperature, they are expected to be particularly vulnerable to global warming. Since the projected rate of climate warming is lower in the tropics than in higher latitudes (IPCC, 2007), impacts of global warming on biodiversity are often assumed to be geographically dependent. There are wide indications that thermal tolerance in different groups of ectotherms is related to the magnitude of temperature variation they normally experience (Janzen 1967, Addo-Bediako et al. 2000, Ghalambor et al. 2006), which should increase with latitude. Most evidence suggests that species from temperate zones should have relatively broader thermal tolerances than tropical species, primarily because they are more tolerant to cold temperatures. Some works are consistent with the prediction that body temperature variability is reduced in the tropics and increases with latitude, for example in salamanders (Feder and Lynch 1982), lizards (Van Berkum 1988) and crabs (Stillman and Somero 2000). Furthermore, tropical ectotherms appear to be thermal specialists with lower acclimation capacity than higher-latitude ectotherms (Van Berkum 1988, Addo-Bediako et al. 2000, Hoffmann et al. 2003a, Ghalambor et al. 2006, Gilman et al. 2006, Deutsch et al. 2008, Calosi et al. 2008).

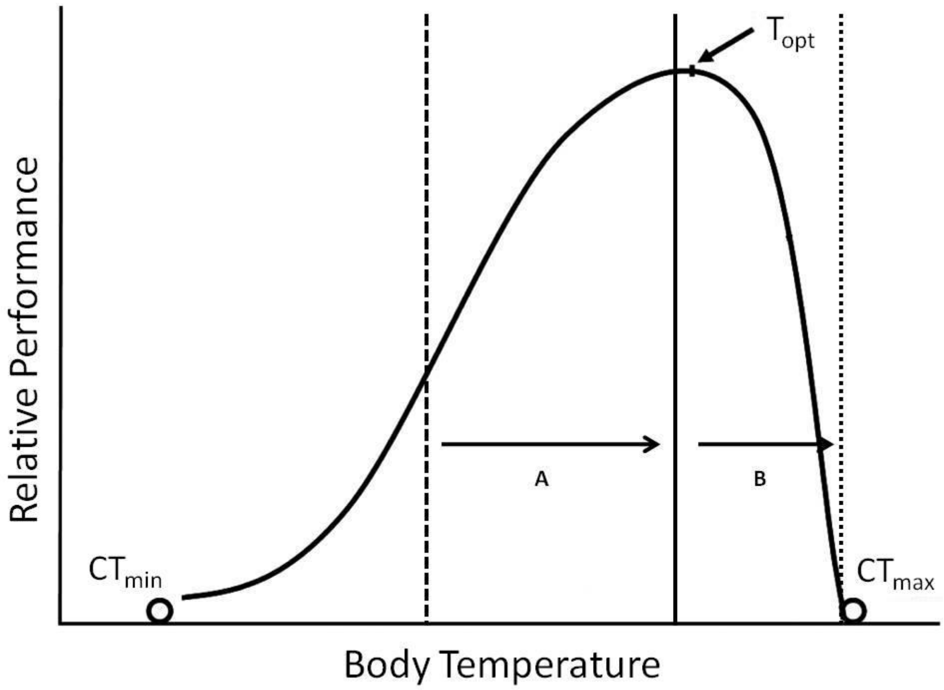


Figure I. General shape of a thermal performance curve, including minimum critical temperature (CT_{min}), optimum temperature (T_{opt}) and, critical thermal maximum (CT_{max}). The three vertical lines represent environmental temperatures. Species that experience a transition in their thermal habitat similar to *B* should be harmed by global warming. In contrast, species that experience a transition in their thermal habitat similar to *A* may benefit from global warming since the environmental temperature moves towards their optimum temperature. In Katzenberger et al. (2012), originally modified from Huey and Stevenson (1979) and Huey and Kingsolver (1989).

In ectotherms, most physiological processes proceed rapidly over a range of body temperatures defining a thermal performance curve or TPC (Huey and Stevenson 1979). This thermal sensitivity curve rises gradually from a minimum critical temperature (CT_{min}), reaches an optimum temperature (T_{opt}), and then falls rapidly to a critical thermal maximum (CT_{max}), as seen in Figure I. Critical thermal limits define the thermal tolerance range of an organism; temperatures either below or above this range of tolerance result in impaired physiological function (Hillman et al. 2009). Given that critical thermal maxima of terrestrial ectotherms varies little much wide ranges of latitude (Addo-Bediako et al. 2000, Ghalambor et al. 2006, Deutsch et al. 2008, Tewksbury et al. 2008) and given many tropical ectotherms live in environments where body temperatures are close to or even above optimal temperatures for performance (Deutsch et al. 2008), these species (constituting the richest faunistic biodiversity in the world) can be at a greater risk of extinction due to global warming than species from higher latitudes. A realistic evaluation of higher geographic impact of global warming in tropical ectotherms should therefore combine both accurate knowledge of specific tolerance of organisms and the environmental temperature they are exposed to.

Recently, Deutsch et al. (2008) provided the first quantitative assessment of this prediction, employing a physiological framework which integrated fitness curves. To assess latitudinal variations in global warming impact on ectotherms, assuming that their basic physiological functions are heavily dependent on environmental temperature, two operative metrics were defined: Warming Tolerance ($WT = CT_{max} – T_{hab}$), as the difference between the organism's critical thermal maximum and its current environmental temperature; and, thermal safety margin ($TSM = T_{opt} – T_{hab}$), as the difference between the organism's thermal optimum (T_{opt}) and its current environmental temperature (T_{hab}). Warming Tolerance is related to the average warming an ectotherm can tolerate before environmental temperature surpasses its upper tolerance limit. As tropical species usually live at higher environmental temperatures, they are expected to have lower Warming Tolerance than high-latitude species. Also, as environmental temperatures are closer to their physiological optimum, tropical species are likely to have narrower TSM. Therefore, any small increase in temperature could trigger a decrease in their performance. However, high-latitude species are exposed to cooler temperatures than their physiological optimum and could therefore benefit from global warming if increasing environmental temperatures do not surpass their optimum temperature. If their optima are surpassed, then high-latitude species would also be negatively affected by global warming.

Most research on thermal adaptation and thermal tolerance in ectotherms has been conducted on a few well-studied model organisms, such as *Drosophila* spp. (e.g., Hoffmann et al. 2003a, 2003b). While this is a fundamental first step, the interest in producing information on focal threatened taxa, in particular those distributed across sensible habitats (e.g., amphibians in tropical and mountain areas), is rising as conservation actions will require a better understanding of the physiological ecology and genetics of these species. Recent studies have started to address this issue by compiling available data and producing new physiological information for several different taxa (Deutsch et al. 2008, Sunday et al. 2011, 2012, 2014, Duarte et al. 2012). In ectotherm vertebrates, most literature available has focused on fish and reptiles. Apart from the first seminal contributions in the 50s - 70s (Brattstrom 1959, 1963, 1968, Hutchison 1961, Heatwole et al. 1965, Mahoney and Hutchison 1969, Lillywhite 1970), thermal physiology research in amphibians has been has only received attention in more recent decades (e. g. Hutchison and Dupré 1992, Rome et al. 1992, Ultsch et al. 1999, Navas et al. 2008, Hillman et al. 2009, Duarte et al. 2012).

Amphibians are considered the most endangered group of vertebrates, with approximately 41% of species being threatened with extinction (Hoffmann et al. 2010). In addition, more than 85% of the current amphibian species are located in the tropics (Wells 2007, Stuart et al. 2008). They have a number of physiological, ecological and life-history characteristics that make them highly susceptible to environmental change such as ectothermy, permeable skin and complex life-cycles (with metamorphosis), an adaptation to the sequential occupation of temporary wetlands (mainly larval stage) and terrestrial environments (adult stage) (Wells 2007). Apart from habitat destruction and degradation, many reported cases of amphibian declines and extirpation of local populations have been caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (Stuart et al. 2004, Lips et al. 2006, Wake and Vredenburg 2008), or similar emergent diseases. Although it is unclear the extent to which global warming could have triggered devastating chytridiomicosis outbreaks (Rohr et al. 2008), there is increasing evidence that epidemic diseases may be driven by changes in the thermal environment (Pounds et al. 2006, Raffel et al. 2006, Bosch et al. 2007, Ruthig 2008). However, since there have been no reports of amphibian mortality associated with extreme heat events (Carey and Alexander 2003a), increasing maximum environmental temperatures have not generally been considered a direct cause of amphibian decline (Collins and Crump 2009), possibly because of the difficult to isolate temperature from other environmental factors in field conditions.

Tadpoles are a great model organism for studying thermal tolerances in ectotherms (Burggren and Warburton 2007). As the larval stage of the amphibian life cycle, tadpoles experience a growth and developmental period where analyzed traits are independent of reproductive condition or gender. Being aquatic, tadpole's body temperature is the same as its surrounding environment (Spotila et al. 1992) and they can not suffer dehydration when exposed to heating as in the terrestrial life stages. However, they can still suffer stress associated with increased environmental temperature, such as a decrease in water dissolved oxygen and subsequently in their aerobic performance (see Pörtner and Knust 2007) or an increase in osmotic stress (Gómez-Mestre and Tejedo 2003, Gómez-Mestre et al. 2004), that may interact with thermal tolerances (Re et al. 2006).

Although tadpoles are capable of behaviourally regulating their body temperatures through selection amongst a range of available environmental temperatures (Noland and Ultsch 1981, Wollmuth et al. 1987, Hutchison and Dupré 1992), their physical environment limits the extent of their thermoregulation (Wu and Kam 2005). This thermoregulatory constraint is particularly evident in tadpoles occupying temporary habitats since short duration ponds are typically shallow with low water volume and can suffer intense heating, especially those receiving direct sunlight, which can result in important daily thermal gradients without thermal stratification. In ponds located in tropical and subtropical environments (with wet summer breeding seasons), tadpoles may be exposed to temperatures over 40°C (Watson et al. 1995, Duarte et al. 2012). During heating waves, expected to increase in frequency along with global mean temperature (IPCC 2007, Diffenbaugh and Ashfaq 2010), tadpoles with limited behavioural thermoregulation possibilities may not be able to escape from hazardous temperatures before ponds dry completely. Furthermore, if pond durations are reduced due to low precipitation, which is predicted for many areas such as Central America and Australia (IPCC 2007), tadpoles will need to metamorphose faster to avoid higher temperatures during pond drying and find a more thermally favourable land microclimate where they would be able to behaviourally thermoregulate (Navas et al. 2007).

Thermal tolerance studies

The analysis of thermal tolerances in amphibians was initially developed by Hutchison (1961) in salamanders and Brattstrom (1968) in anurans. Interestingly, Brattstrom's study included comparative data of CT_{max} for 53 species of frogs, over a latitudinal and altitudinal gradient in North and Central America, and he found that CT_{max} varied at both species and population levels. For most anuran larvae, CT_{max} was determined to fall between 38°C and 42°C (Brattstrom 1968, Ultsch et al. 1999, Bury 2008, Navas et al. 2010). Several studies have demonstrated within-species variation in heat tolerance (Hutchison 1961, Brattstrom 1968, 1970, Miller and Packard 1977, Hertz et al. 1979, Garland Jr. and Adolph 1991, Gvoždík and Castilla 2001, Winne and Keck 2005, Huang and Tu 2008); it can also vary adaptively amongst populations (Skelly and Freidenburg 2000, Wu and Kam 2005).

The critical thermal maximum is affected by several factors. During ontogeny, CT_{max} of tadpoles can drop 3-4°C when close to metamorphic climax (Floyd 1983) while acclimation to higher temperatures may increase CT_{max} up to 4°C (Brattstrom, 1968; Navas et al., 2008). Other factors that influence CT_{max} estimates include ramping rate (Terblanche et al. 2007, Chown et al. 2009b, Mitchell and Hoffmann 2010), selection of endpoint (Lutterschmidt and Hutchison 1997a, 1997b) and photoperiod (Mahoney and Hutchison 1969). CT_{max} also exhibits phylogenetic signal and differences between amphibian lineages can be found both in adult stage (Navas et al. 2008) and tadpoles (Duarte et al. 2012).

Some debate still exists whether CT_{max} is dependent on latitude or not. Analysis on insects revealed no geographical trend (Addo-Bediako et al. 2000) but in amphibians the analysis of Brattstrom (1968) data set is inconclusive; Snyder and Weathers (1975) found a significant decline in CT_{max} with increasing latitude ($r=0,70$; $p<0,05$) whereas the re-analysis of Ghalambor et al. (2006) showed that the trend was not significant ($p > 0,70$). The recent work of Duarte et al. (2012), comparing two subtropical communities with different thermal regimes (the hot and semi-arid Chaco region and the humid Misiones province, both in Argentina) and one temperate community (Iberian Peninsula), showed that CT_{max} is positively correlated with maximum environmental temperature and therefore species from the Chaco region had higher CT_{max}.

Thermal sensitivity studies

Thermal sensitivity and optimal temperature in locomotor performance have been the subject of recent ectotherm studies (e.g., Bauwens et al. 1995, Claussen et al. 2000), including amphibians (Rome et al. 1992, Wilson 2001, Gomes et al. 2002, Anholt et al. 2005, Dayton et al. 2005, Gvoždík and Van Damme 2006, 2008, Arendt 2009, 2010).

As animals often perform under sub-optimal environmental conditions (Huey et al. 1989), there has been increasing interest not only on maximal performance capacity but also on the shape and position of the reaction norm (thermal performance curve) describing the environmental dependence of physiological performance (Schlichting and Pigliucci 1998, Angilletta et al. 2003, Angilletta 2006, Kingsolver et al. 2007, Gvoždík and Van Damme 2008).

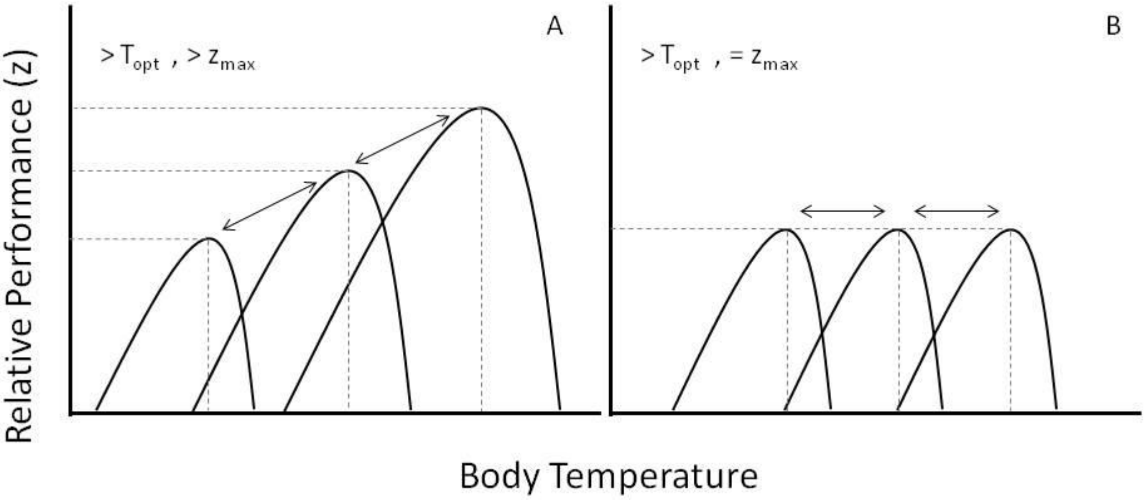


Figure II. The thermodynamic hypothesis or "hotter (warmer) is better” (A) predicts that species with higher optimum temperatures (T_{opt}) will have relatively high maximum performance (z_{max}). The perfect-compensation hypothesis (B) predicts that maximum performance will be independent of optimum temperature. Modified from Frazier et al. (2006).

If adaptation is unable to overcome the rate-depressing effects of low temperature, organisms adapted to lower temperatures are predicted to have lower maximum performances (e.g., sprinting speeds and fitness) than those adapted to higher temperatures (Bennett 1987). In terms of continuous reaction norms, the “hotter is better” hypothesis would then predict a positive correlation between an organism’s optimal temperature and its maximum performance at the optimal temperature (Hamilton 1973, Huey and Kingsolver 1989, Savage et al. 2004, Frazier et al. 2006, Knies et al. 2009; Figure II.A). In contrast, if organisms living at low temperatures can compensate for rate-depressing effects (Huey and Kingsolver 1989), then organisms inhabiting low temperatures should achieve the same maximum performances as those living at high temperatures (Frazier et al. 2006, Knies et al. 2009; Figure II.B). Several comparative studies between species (or higher taxa) have shown strong support for “hotter is better” (Eppley 1972, Bauwens et al. 1995, Rehfeldt et al. 2002, Heilmayer et al. 2004, Frazier et al. 2006; see Angilletta et al. 2010). However, support for this hypothesis is quite mixed (or yield weaker results) for some aspects of performance, such as locomotion (Kingsolver 2009, Angilletta et al. 2010). Furthermore, if the “hotter is better” pattern that characterizes comparisons between species reflects limitations on adaptation of reaction norms, then intraspecific comparisons should also reflect those limitations and present that same pattern (Knies et al. 2009). Currently, experiments that studied the “hotter is better” hypothesis in an intraspecific context have produced mixed results, with some observing the pattern (e.g., Knies et al. 2009) and others not (e.g., Izem and Kingsolver 2005).

Another common assumption is that maximal performance or fitness is inversely related to temperature range, the ‘‘Jack-of-all-temperatures is a master of none’’ hypothesis (Huey and Hertz 1984). As a result of this thermal specialist-generalist trade-off, selection for an increased temperature range is expected to incur in a reduction in maximal performance and selection for maximal performance is expected to reduce an organism’s temperature range (Levins 1968, Huey and Slatkin 1976). If such trade-offs exist, then “hotter is better” can be achieved only through a narrowing of reaction norms, resulting in “hotter is narrower”. However, despite being a common assumption in evolutionary models of thermal adaptation (Lynch and Gabriel 1987, Gilchrist 1995), there is little empirical support for generalist-specialist trade-offs (Huey and Hertz 1984, Angilletta et al. 2002, Yamahira et al. 2007). If generalist-specialist trade-offs do not constrain adaptation to temperature, then hotter is better could generate a higher and broader reaction norm, increasing the temperature range (Knies et al. 2009). Although some authors have approached this question (Gvoždík and Van Damme 2008), there is still little information on the proportions of thermal generalist-specialist species, both in tropical and temperate communities.

Thesis structure and planning

This doctoral thesis is divided in two different approaches; one with a broader approach to the thesis theme and a second consisting of several more specific approaches. The first approach, which is also the main objective of this thesis, aims to extend current knowledge on amphibian optimum temperatures (including thermal performance curves) and assess whether tropical amphibians species (living normally under higher environmental temperatures) are more vulnerable to global warming than temperate species (living normally under colder environmental temperatures). Chapter 1, **"Coping with increasing environmental temperatures: how vulnerable are amphibians to climate change?"**, addresses this question by studying the thermal physiology of tadpoles from over 70 species, encompassing different biomes and countries. A large dataset was created by determining the thermal physiology of these species, with the use of thermal performance curves, and by measuring each species' environmental temperatures. Using the metrics established in Deutsch et al. (2008), this dataset allowed the identification of amphibian species and biomes that are more vulnerable to climate changes.

Forecasting biological responses to current climatic changes emphasizes the necessity of understanding species thermal physiology and to assess their potential to face these changes via either plasticity or evolution. The second approach addresses the plasticity of thermal physiology of amphibian species, with a particular focus on thermal performance curves, and what this variation means to the vulnerability assessment made in chapter 1. Large global comparative studies have additional difficulties, such as logistics and time constraints, which can limit the outcome of the work. Since only one population per species was used in chapter 1 to keep species sampling and testing on a reasonable scale, it is important to determine how much variation exists within a species and if a single population can be representative of that species' thermal physiology and vulnerability estimates. Chapter 2, **"Thermal physiology variation and vulnerability to thermal stress in *Pelodytes* spp. populations from the Iberian Peninsula"**, is a study on variation in CT_{max} and thermal performance curves (including optimum temperature) of populations from two different *Pelodytes* species, and investigates whether their thermal physiology is phylogenetic constrained or if there is local adaptation to the thermal environment. It also includes an evaluation of each population's susceptibility to acute and chronic thermal changes (increasing environmental temperatures) by calculating their Warming Tolerance and their Thermal Safety Margins respectively, again by applying the metrics defined in Deutsch et al. (2008).

Studies like those conducted in the first two chapters of this thesis are usually performed under laboratory conditions. Although they give very important information on the thermal physiology of species, it is also important to keep in mind that organisms are exposed to a set of environmental conditions that can vary. Hence, there is a need to understand how a species' physiology varies through space and time and assess how changes in physiological function due to environmental changes may interact with phenotypic changes caused by other types of environmental variation. Amphibian larvae are well known for expressing environmentally induced phenotypes, but relatively little is known about how these responses might interact with changing temperatures and the thermal physiology of organisms. This question is addressed in chapter 3, entitled **"Swimming with predators and pesticides: How environmental stressors affect the thermal physiology of tadpoles"**, where the thermal physiology of grey treefrog tadpoles (*Hyla versicolor*) is studied by determining whether exposures to predator cues and an herbicide (Roundup®) can alter the tadpole's critical maximum temperature (CT_{max}) and swimming speed across a range of temperatures. This provides estimates of optimal temperature (T_{opt}) for swimming speed and the shape of the thermal performance curve (TPC) and highlights the importance of considering the plastic responses of CT_{max} and TPC to different inducing environments when forecasting biological responses to global warming.

As mentioned before, amphibians have a number of physiological, ecological and life-history characteristics that make them highly susceptible to environmental change, including a complex life-cycle (Wells 2007). Metamorphosis occurs in the amphibian's life-cycle, and it is presumed to be an adaptation to the sequential occupation of temporary wetlands and terrestrial environments (Wells 2007). However, for organisms such as amphibians that experience different selective environments during their development, genetic correlations between ontogenetic stages can mean that selection on a trait at one stage induces maladaptive change in the same trait at other stages (Watkins 2001). Hence, metamorphosis is commonly seen as being beneficial since it may break the developmental and genetic relationships between traits expressed at different stages (Ebenman 1992, Moran 1994), and thereby allow the pre- and postmetamorphic stages to adapt independently to their respective environments (Watkins 2001) - the adaptive decoupling hypothesis (Moran 1994, Watkins 2001).

Since thermal physiology traits such as CT_{max} and optimum temperature have been shown to be evolutionarily correlated with environmental temperature (Chapter 1; Duarte et al., 2012), thus reflecting species adaptation to their thermal habitat, chapter 4 of this thesis, **"Vulnerability to climate change across life-stages in amphibian species"**, is a study on two stages of the amphibian life-cycle to determine if adaptation to the thermal environment in one stage can result in maladaptive traits in another stage. Here, the thermal physiology of the tadpole and juvenile stages is compared, using thermal performance curves to estimate optimum temperature and other related physiology traits. This also allows the comparison of Thermal Safety Margins of tadpoles and juveniles to determine if there is a life-stage that may be more vulnerable to suffer long-term chronic effects from increasing environmental temperatures, such as diminished physiological, developmental or behavioral performance at higher temperatures, and would determine if estimates of vulnerability to climate change in a life-stage can be extrapolated to the whole life-cycle of the amphibian species.

Finally, apart from the discussion in each chapter, the main results are compiled in **"General Conclusions"** and summarize the most important contributions of this doctoral thesis to current questions addressed by the scientific community.

The General Introduction is partially published in:

Katzenberger, M., M. Tejedo, H. Duarte, F. Marangoni, and J. F. Beltrán. 2012. Tolerância e sensibilidade térmica em anfíbios. Revista da Biologia 8:25–32.

CHAPTER 1

Coping with increasing environmental temperatures: how vulnerable are amphibians to climate change?

Resumen

Durante las próximas décadas de cambio climático global, se prevén temperaturas medias más altas, una mayor frecuencia de fenómenos climáticos extremos y cambios en los patrones de precipitación. Como los impactos ecológicos dependerán de la magnitud y del patrón del cambio climático y de la sensibilidad térmica de los organismos, para identificar las especies y las comunidades que podrán estar expuestas a un mayor riesgo de estrés fisiológico es crucial entender mejor los mecanismos relacionados con la función fisiológica y con el desempeño ecológico. En este trabajo, estudiamos la fisiología térmica de larvas /renacuajos de 71 especies de anfibios, de diferentes regiones climáticas, mediante la estimación de sus temperaturas óptimas usando curvas de desempeño térmico y compilando información sobre sus temperaturas críticas máximas. Además, determinamos la tolerancia al calentamiento y las márgenes de seguridad térmica para esas especies con el objetivo de evaluar su vulnerabilidad al calentamiento global. Nuestros resultados indicaron que las especies de las comunidades tropicales y subtropicales son más vulnerables a sufrir tanto estrés agudo (tolerancia al calentamiento) como estrés crónico (márgenes de seguridad térmica) debido a las mayores temperaturas ambientales locales. Por otra parte, obtuvimos valores positivos de tolerancia al calentamiento y márgenes de seguridad térmica, indicando qué generalmente las temperaturas ambientales máximas y medias no superan la temperatura crítica máxima y la temperatura óptima, respectivamente. Aún así, nuestros datos también muestran que varias especies, incluyendo algunas de las latitudes más altas, ya experimentan temperaturas muy cercanas o incluso por encima de sus temperaturas óptimas de desempeño. Por lo tanto, algunas especies de regiones templadas también pueden verse negativamente afectadas por el calentamiento global. Estos resultados son especialmente preocupantes ya que la mayor parte de la biodiversidad de los anfibios se encuentra en las regiones tropicales y subtropicales. Además, dado que la dispersión de esta Clase se produce sólo en la etapa terrestre, las larvas de anfibios tendrán que confiar en su capacidad de aclimatar su fisiología térmica y comportamientos de termorregulación para hacer frente a los cambios térmicos del ambiente.

Palabras clave: temperatura óptima, cambios climáticos, curvas de desempeño térmico, latitud, temperatura crítica máxima, tolerancia al calentamiento, márgenes de seguridad térmica.

Abstract

Global climate change over the coming decades is predicted to cause higher mean temperatures, an increased frequency of extreme climatic events, and changes in precipitation patterns. As ecological impacts will depend on both the magnitude and pattern of climate change and the thermal sensitivity of the organisms in question, it is crucial to better understand the mechanisms relating physiological function and ecological performance and to identify the species and communities currently exposed to higher risk of suffering physiological stress. We studied the thermal physiology of tadpoles from 71 amphibian species, from different climatic regions, by estimating their optimum temperatures using thermal performance curves and compiling information on their critical upper thermal limit. Furthermore, we determined Warming Tolerance and Thermal Safety Margins for these species to assess their vulnerability to global warming. Our results indicate that species from tropical and subtropical warm communities are more vulnerable to suffer from acute (Warming Tolerance) and chronic stress (Thermal Safety Margins) due to higher local environmental temperatures. Furthermore, we obtained positive Warming Tolerance and Thermal Safety Margins estimates, indicating that generally maximum and average environmental temperatures do not surpass critical thermal maximum and optimum temperature, respectively. However, our data also shows that several species, including some from higher latitudes, already experience temperatures very close or even above their optimal performance temperatures. Hence, some species from temperate regions may also be negatively affected by global warming. This findings are particularly worrying since most of the amphibian biodiversity is located the tropical and subtropical regions. Moreover, since dispersal only occurs in the terrestrial stage, amphibian tadpoles will have to rely on their ability to acclimate their thermal physiology and behaviour changes (thermoregulation) to cope with the changing thermal environment.

Keywords: optimum temperature, climate change, thermal performance curves, latitude, critical thermal maximum, Warming Tolerance, Thermal Safety Margins

Introduction

During the past century, the Earth has experienced a mean increase in temperature of 0.6 °C, reaching up to 4 °C in the most northern latitudes, which has already caused significant changes in species' distribution patterns, the structure and functioning of ecosystems and the timing of biological processes (Root et al. 2003, Parmesan 2006). IPCC's (2013) predicts a five-fold increase in warming rate for the coming decades, including the increase of extreme climatic events (Schär et al. 2004, Diffenbaugh and Ashfaq 2010) and alteration of global precipitation patterns. Recent works argue that an extinction process is already underway and project further erosion of diversity associated with climate changes (Sinervo et al. 2010). As ecological impacts will depend on both the magnitude and pattern of climate change and the thermal sensitivity of the organisms in question (Helmuth et al. 2005, Tewksbury et al. 2008, Kingsolver 2009), it is crucial to better understand the mechanisms relating physiological function and ecological performance and to identify the species and communities currently exposed to higher risk of suffering physiological stress (Somero 2005, Helmuth et al. 2010).

Ectotherms constitute the majority of current biodiversity and their basic physiological functions (e.g., locomotion, growth, or reproduction) are strongly influenced by environmental temperature, which makes them especially vulnerable to climate warming. Low-latitude tropical ectotherms usually experience warmer environmental temperatures than higher latitude temperate ectotherms (Clarke and Gaston 2006, Deutsch et al. 2008, Tewksbury et al. 2008). If differences between their thermal limits do not compensate for the differences in their thermal environmental, then low-latitude tropical ectotherms should experience environmental temperatures closer to their upper thermal thresholds and optimal temperatures than temperate ectotherms (Addo-Bediako et al. 2000, Compton et al. 2007, Deutsch et al. 2008, Huey et al. 2009, Sunday et al. 2011). Based on these assumptions, recent studies suggest that tropical ectotherms from lower latitudes are more vulnerable to environmental warming than their temperate counterparts (Stillman and Somero 2000, Compton et al. 2007, Deutsch et al. 2008, Tewksbury et al. 2008, Huey et al. 2009, Dillon et al. 2010).

Amphibians are considered the most globally threatened group of vertebrates (Wake and Vredenburg 2008, Hoffmann et al. 2010). Although some indirect factors associated or reinforced by global warming have been identified, such as emerging infectious diseases, changes in the moisture content of terrestrial environments or shortening of pond hydroperiod (Pounds et al. 2006, Raffel et al. 2006, Bosch et al. 2007, Ruthig 2008, McMenamin et al. 2008), the direct effect of increasing environmental temperatures has generally been neglected as a direct causal factor of amphibian decline (Collins and Crump 2009). So far, no evidence of lethal episodes of acute warming on any amphibian population has been reported (Carey and Alexander 2003b) and the chronic effects of exposure to warmer temperatures may be difficult to distinguish from other factors. Nevertheless, some amphibian communities (e.g. tropical montane salamanders with narrow distributions) are thought to be prone to extinction (Wake and Vredenburg 2008) due to, among other factors, warming-induced physiological depression (Bernardo and Spotila 2006). Tropical and subtropical amphibians, which represent 80–90% of extant amphibian fauna (Wells 2007), have relatively high critical thermal limits (Brattstrom 1968, Snyder and Weathers 1975; but see Feder and Lynch 1982). However, depending on the type of habitat, they can be exposed to higher environmental temperatures, causing their tolerance to warming to be rather small (Duarte et al. 2012).

Previous works in amphibian thermal physiology have focused on species' thermal limits to evaluate their vulnerability to global warming, estimating the acute effect of increasing environmental temperatures (Deutsch et al. 2008, Duarte et al. 2012, Sunday et al. 2014). Here we evaluate the vulnerability of amphibian species to suffer long-term chronic effects from increasing environmental temperatures, such as diminished physiological, developmental or behavioural performance at higher temperatures. As amphibians are ectotherms, their ability to perform basic physiological functions at different temperatures is described by a thermal performance curve, which indexes the direct effect of temperature on organism fitness (Huey and Stevenson 1979, Frazier et al. 2006), providing a physiological framework for elucidating a fundamental component of the impact of global climate change in a spatially explicit and empirically constrained way (Deutsch et al. 2008). Hence, we studied the locomotor performance of amphibian tadpoles of species from different communities by using thermal performance curves to estimate species' optimum temperatures and their Thermal Safety Margins.

As optimum temperature can be related to the critical thermal maximum (CT_{max}) (Huey et al. 2009), we expect tropical and subtropical amphibians to have higher optimum temperatures than temperate species. Since CT_{max} has been shown to be habitat dependent (Duarte et al. 2012), we also expect optimum temperature to be evolutionarily correlated with environmental temperature, showing species' adaptation to their thermal habitat. Furthermore, we hypothesize that low latitude amphibian species will have narrower Thermal Safety Margins because current average environmental temperature is closer to their optimum temperature than in higher latitude species, as seen in other taxonomic groups (Deutsch et al. 2008, Huey et al. 2009).

Methods

For this work, we sampled larvae of 71 amphibian species from different locations in five countries: Argentina, Brazil, USA, Portugal and Spain (Annexe I-A). Field sampled larvae were transported to the laboratory at each particular study site (Argentina, CECOAL-CONICET, Corrientes 2009 and FCEQyN-UNaM, Posadas, 2010-2011; Spain (Iberian Peninsula), EBD-CSIC, Sevilla 2009-2011; USA, Pennsylvania, Pymatuning Laboratory of Ecology, 2010-2011; Brazil, Universidade Estadual de Santa Cruz, UESC, Ilhéus, Bahia 2011-2012). All larvae were maintained at similar conditions, with constant room temperature of 20 °C, photoperiod of 12L:12D and fed rabbit chow *ad libitum*. We followed the updated Köppen-Geiger climate classification (Kottek et al. 2006) to group species according to the main climates and precipitation conditions (Annexe I-A), using as reference each species’ collection site. These climatic groups were then used in subsequent analysis.

To assess the environmental temperatures to which these species are exposed during their breeding season, we considered two approaches: a) mapping a set of global climate layers (climate grids) available in WorldClim (Hijmans et al. 2005) and extracting temperature data for the collection sites using QGIS software; and b) recording water temperature every 5-15 min, using a HOBO Pendant® temperature dataloggers placed in each collection site at the deepest part of the pond, only when it held water (during breeding season). In both cases we extracted estimates of pond T_{avg} (average temperature), T_{max} (maximum temperature), T_{min} (minimum temperature) and, DTF (diel temperature fluctuation; Annexe I-B). For

Spea multiplicata and *Scaphiopus couchii*, datalogger information was extrapolated from Morey and Reznick (2004).

Thermal physiology and vulnerability assessment

We estimated thermal sensitivity of tadpoles using thermal performance curves (TPCs) based on maximum locomotor performance (burst swimming speed) at different water temperatures. Locomotor performance has been employed as a good proxy to estimate optimal temperatures in ectotherms since it may correlate with fitness (Jayne and Bennett 1990, Le Galliard et al. 2004, Husak 2006). To determine burst swimming speed (i.e. burst speed), tadpoles were placed individually on a portable thermal bath (patent license ES 2372085), which consists of an opened cross section methacrylate tube (1 m long x 6 cm wide x 3 cm deep) filled with water of a given temperature. We then gently prodded the tadpole with a thin stick to stimulate swimming. Each trial was recorded using a digital camera (30 frames/s) positioned above the tube (JVC Everio GZ-MG505). TPCs were defined using a set of temperatures that were tested in a random order. As species differ in their range of performance, to obtain a complete performance curve, temperature sets were adjusted by adding colder or hotter test temperatures when required, as seen in Annexe I-C. Prior to swimming, tadpoles were submitted for approximately an hour to the test temperature.

We used Measurement in Motion v3.0 software (Learning in Motion 2004) to estimate burst speed over three frames (0.1 s) after the tadpole started to move by measuring the distance the centre of mass moved between frames (Arendt 2009, 2010). We then considered the fastest speed of three or more bouts as our final measure of burst speed. Since sprint speed may scale with body size (Gvoždík and Van Damme 2006) and body size may confound the effect of speed on escape success (Van Damme and Van Dooren 1999), we used size-corrected burst speed (tadpole total length) for constructing thermal performance curves (Annexe I-C). To describe the TPCs for burst speed, we used the Template Mode of Variation method (TMV; Izem and Kingsolver 2005). Since we tested tadpole performance at several temperatures, we assumed that the common template curve was a fourth-degree polynomial, as in previous studies (Gvoždík and Van Damme 2008, Katzenberger et al. 2014). For each species, we also calculated maximum performance (z_{max}) and performance breadth B_{80}, (range of temperatures at which performance values exceed 80% of the maximum; Huey and Stevenson 1979).

We assessed each species' vulnerability to acute and chronic thermal changes, using two metrics defined in Deutsch et al. (2008): Warming Tolerance (WT), which is the difference between species' upper critical thermal limit (CT_{max}) and its current (maximum) environmental temperature (T_{max}); and Thermal Safety Margins (TSM), which is the difference between the species' thermal optimum (T_{opt}) and its current (mean) environmental temperature (T_{avg}). We calculated WT and TSM using both datalogger and WorldClim environmental temperature information. CT_{max} estimates used in WT calculations were obtained from available literature (Duarte et al. 2012, Gutierrez-Pesquera et al. unpublished data).

Statistical and phylogenetic comparative analyses

Because the data were collected across multiple species, all statistical analyses were undertaken incorporating phylogenetic information (Felsenstein 1985, Garland Jr. et al. 1992). Hence, we extracted a phylogenetic tree for the sampled species from Pyron and Wiens (2011), keeping both topology and branch lengths estimated in that work. To evaluate the correlations between physiological measurements and environmental temperature data, we used phylogenetic generalized least squares (PGLS) analysis under a Brownian motion model of evolution, in package *caper* (Orme et al. 2013) for R (R Development core team 2014). We also used *phylosig* in package *phytools* (Revell 2012) for R to determine if thermal physiology traits exhibit phylogenetic signal (Pagel's λ).

To analyze the influence of climatic region on the thermal physiology and the measurements of species vulnerability to environmental changes, we used phylogenetic ANOVA models in package *phytools* (Revell 2012) for R. ANOVA models were followed by post-hoc pairwise comparisons of means among groups with p-values adjusted for multiple comparisons using FDR correction (Benjamini and Hochberg 1995). We also compared WT and TSM estimates when using datalogger or WorldClim environmental information, by performing paired *t*-tests (WT and TSM separately). All analysis reported were performed in R (R Development core team 2014), except when mentioned otherwise, and were conducted on a significance level of $\alpha=0.05$. Since we incorporated phylogenetic information, taxa that could not be identified to a species level were excluded from the analysis.

Results

Using the TMV method, we estimated thermal performance curves for 71 species (Annexe I-D) and determined the physiology traits associated with them (T_{opt}, z_{max} and B_{80}; Annexe I-E). Species with higher optimum temperature showed narrower performance breadths ($\lambda = 0.98$, $\beta = -0.14 \pm 0.05$, $t = -2.66$, $p = 0.010$) and maximum performance was not related to either optimum temperature or performance breadth. However, maximum performance was related to latitude while optimum temperature and performance breadth were not (Table 1.1). Furthermore, upper and lower limits of B_{80} are related amongst themselves ($\lambda = 0.91$, $\beta = 0.15 \pm 0.05$, $t = 3.38$, $p = 0.001$). CT_{max} estimates were related to optimum temperature ($\lambda = 0.82$, $\beta = 0.50 \pm 0.05$, $t = 10.15$, $p < 0.001$) and both upper ($\lambda = 0.60$, $\beta = 0.67 \pm 0.06$, $t = 11.06$, $p < 0.001$) and lower limits of B_{80} ($\lambda = 0.87$, $\beta = 0.15 \pm 0.04$, $t = 3.74$, $p < 0.001$) but not B_{80} and maximum performance.

Optimum temperature was related to most of the environmental temperature measurements taken (the only exception being minimum environmental temperature T_{min}), either using datalogger or WorldClim data (Table 1.1), increasing with maximum temperature (T_{max}), average temperature (T_{avg}) and diel temperature fluctuation (DTF_{avg} and DTF_{annual}). When compared to datalogger environmental information, maximum performance increased with T_{min} and decreased with T_{max} and DTF_{avg}, while when using WorldClim environmental information only a decrease with DTF_{annual} was evident. Although no relation between performance breadth (B_{80}) and the environmental temperature measurements taken was found, both upper and lower limits of B_{80} (B_{80max} and B_{80min}) increased with T_{max}, T_{avg} and, DTF. Altitude was not related to any of the thermal physiology measurements taken (Table 1.1).

Table 1.1. PGLS results for the relation between thermal physiology traits and environmental temperature measurements. Significant relations are shown in bold.

	T_{opt}	B_{80}	z_{max}	B_{80max}	B_{80min}
latitude	$\lambda = 0.98, \beta = 0.00 \pm 0.04$ $t = 0.10, p = 0.922$	$\lambda = 0.00, \beta = -0.01 \pm 0.05$ $t = -0.23, p = 0.820$	**$\lambda = 0.76, \beta = -0.21 \pm 0.06$** **$t = -3.58, p < 0.001$**	$\lambda = 0.96, \beta = 0.04 \pm 0.03$ $t = 1.17, p = 0.247$	**$\lambda = 0.74, \beta = 0.14 \pm 0.07$** **$t = 2.02, p = 0.047$**
altitude	$\lambda = 0.98, \beta = 0.12 \pm 0.39$ $t = 0.30, p = 0.769$	$\lambda = 0.00, \beta = 0.23 \pm 0.85$ $t = 0.27, p = 0.789$	$\lambda = 0.74, \beta = -1.03 \pm 0.86$ $t = -1.19, p = 0.239$	$\lambda = 0.96, \beta = 0.01 \pm 0.36$ $t = 0.03, p = 0.974$	$\lambda = 0.46, \beta = -0.23 \pm 1.01$ $t = -0.23, p = 0.821$
datalogger					
T_{max}	**$\lambda = 0.95, \beta = 0.19 \pm 0.05$** **$t = 4.11, p < 0.001$**	$\lambda = 0.00, \beta = -0.04 \pm 0.08$ $t = -0.58, p = 0.566$	**$\lambda = 0.79, \beta = -0.22 \pm 0.08$** **$t = -2.67, p = 0.010$**	**$\lambda = 0.73, \beta = 0.22 \pm 0.04$** **$t = 6.06, p < 0.001$**	**$\lambda = 0.00, \beta = 0.33 \pm 0.09$** **$t = 3.88, p < 0.001$**
T_{avg}	**$\lambda = 0.84, \beta = 0.32 \pm 0.07$** **$t = 4.86, p < 0.001$**	$\lambda = 0.0, \beta = 0.00 \pm 0.09$ $t = 0.03, p = 0.973$	$\lambda = 0.79, \beta = 0.13 \pm 0.12$ $t = 1.08, p = 0.284$	**$\lambda = 0.84, \beta = 0.27 \pm 0.05$** **$t = 5.26, p < 0.001$**	**$\lambda = 0.00, \beta = 0.33 \pm 0.10$** **$t = 3.34, p = 0.001$**
T_{min}	$\lambda = 0.97, \beta = 0.04 \pm 0.05$ $t = 0.71, p = 0.481$	$\lambda = 0.0, \beta = 0.03 \pm 0.07$ $t = 0.49, p = 0.629$	**$\lambda = 0.78, \beta = 0.24 \pm 0.08$** **$t = 2.99, p = 0.004$**	$\lambda = 0.96, \beta = 0.03 \pm 0.04$ $t = 0.68, p = 0.496$	$\lambda = 0.61, \beta = -0.08 \pm 0.09$ $t = -0.87, p = 0.385$
DTF_{avg}	**$\lambda = 0.97, \beta = 0.16 \pm 0.07$** **$t = 2.41, p = 0.019$**	$\lambda = 0.00, \beta = -0.07 \pm 0.12$ $t = -0.62, p = 0.536$	**$\lambda = 0.65, \beta = -0.38 \pm 0.12$** **$t = -3.15, p = 0.003$**	**$\lambda = 0.84, \beta = 0.18 \pm 0.06$** **$t = 3.07, p = 0.003$**	**$\lambda = 0.56, \beta = 0.36 \pm 0.13$** **$t = 2.79, p = 0.007$**
WorldClim					
T_{max}	**$\lambda = 0.95, \beta = 0.42 \pm 0.07$** **$t = 5.69, p < 0.001$**	$\lambda = 0.00, \beta = -0.10 \pm 0.10$ $t = -0.99, p = 0.328$	$\lambda = 0.81, \beta = -0.07 \pm 0.14$ $t = -0.48, p = 0.634$	**$\lambda = 0.85, \beta = 0.36 \pm 0.06$** **$t = 6.27, p < 0.001$**	**$\lambda = 0.00, \beta = 0.51 \pm 0.11$** **$t = 4.69, p < 0.001$**
T_{avg}	**$\lambda = 0.93, \beta = 0.29 \pm 0.07$** **$t = 4.03, p < 0.001$**	$\lambda = 0.00, \beta = -0.06 \pm 0.08$ $t = -0.82, p = 0.418$	$\lambda = 0.79, \beta = 0.15 \pm 0.12$ $t = 1.26, p = 0.214$	**$\lambda = 0.95, \beta = 0.16 \pm 0.06$** **$t = 2.61, p = 0.011$**	**$\lambda = 0.00, \beta = 0.30 \pm 0.09$** **$t = 3.43, p = 0.001$**
T_{min}	$\lambda = 0.98, \beta = 0.00 \pm 0.05$ $t = 0.10, p = 0.923$	$\lambda = 0.00, \beta = -0.02 \pm 0.06$ $t = -0.41, p = 0.683$	$\lambda = 0.78, \beta = 0.15 \pm 0.08$ $t = 1.92, p = 0.059$	$\lambda = 0.96, \beta = -0.04 \pm 0.04$ $t = -0.95, p = 0.346$	$\lambda = 0.50, \beta = -0.02 \pm 0.09$ $t = -0.20, p = 0.844$
DTF_{annual}	**$\lambda = 0.98, \beta = 0.26 \pm 0.09$** **$t = 3.10, p = 0.003$**	$\lambda = 0.00, \beta = -0.20 \pm 0.17$ $t = -1.16, p = 0.251$	**$\lambda = 0.62, \beta = -0.54 \pm 0.17$** **$t = -3.09, p = 0.003$**	**$\lambda = 0.90, \beta = 0.32 \pm 0.08$** **$t = 3.98, p < 0.001$**	**$\lambda = 0.74, \beta = 0.77 \pm 0.19$** **$t = 4.13, p < 0.001$**

When comparing climatic regions, species from dry climates (B) had higher optimum temperatures than those from temperate (C) or continental (D) climates (Table 1.2). In particular, dry semiarid species (BS) showed higher optimum temperature than those from the Mediterranean (Cs) and hot summer continental (Df) climates. Furthermore, species from humid subtropical climates with precipitation year round or dry winters (Cf and Cw respectively) also had higher optimum temperature than Mediterranean (Cs) species (Table 1.3). Species from tropical climates (A) had higher maximum performance than those from temperate regions (C) and dry climates (B) (Table 1.2). However, when evaluating on a finer scale, differences between groups are less evident with only some tropical tadpoles (Af) showing a marginally significant tendency to be faster than those from dry semiarid regions (BS) and humid subtropical climate with precipitation year round (Cf) (Table 1.3).

Table 1.2. Phylogenetic ANOVAs and pots-hoc results for comparison of thermal physiology traits amongst Köppen-Geiger groups (first letter only), including mean ± SE of traits per group. Significant results are marked in bold.

koppen (N)	**T_{opt}**		**B_{80}**		**z_{max}**		**B_{80max}**		**B_{80min}**	
A (15)	33.2 ± 0.2		16.7 ± 0.5		19.8 ± 0.9		36.8 ± 0.3		20.1 ± 0.5	
B (6)	36.2 ± 0.3		17.0 ± 2.4		12.2 ± 2.3		40.2 ± 0.6		23.2 ± 2.1	
C (37)	31.5 ± 0.7		14.8 ± 0.6		15.0 ± 0.6		36.7 ± 0.5		21.9 ± 0.9	
D (8)	30.8 ± 0.8		17.6 ± 1.4		16.1 ± 0.6		37.5 ± 0.6		19.9 ± 1.3	
	$F_{3,63} = 5.91$ **$p = 0.018$**		$F_{3,63} = 2.62$ $p = 0.225$		**$F_{3,63} = 8.09$** **$p = 0.002$**		**$F_{3,63} = 4.56$** **$p = 0.04$**		$F_{3,63} = 1.17$ $p = 0.63$	
Post-hoc	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*
A - B	-2.56	0.110	-0.66	0.840	**4.34**	**0.003**	**-3.12**	**0.051**	-1.45	0.630
A - C	1.28	0.473	1.62	0.568	**4.11**	**0.003**	0.23	0.883	-1.30	0.630
A - D	1.75	0.464	-0.75	0.840	2.22	0.191	-0.65	0.857	0.09	0.965
B - C	**3.80**	**0.012**	1.94	0.399	-1.77	0.191	**3.63**	**0.018**	0.65	0.678
B - D	**3.75**	**0.012**	-0.05	0.973	-1.96	0.191	2.21	0.220	1.35	0.630
C - D	0.96	0.473	-2.12	0.399	-0.73	0.591	-0.90	0.759	1.11	0.630

Table 1.3. Phylogenetic ANOVAs and pots-hoc results for comparison of thermal physiology traits amongst Köppen-Geiger groups (first two letters), including mean ± SE of traits per group. Significant results are marked in bold.

koppen (N)	**T_{opt}**		**B_{80}**		**z_{max}**		**B_{80max}**		**B_{80min}**	
Af (12)	33.4 ± 0.2		16.7 ± 0.6		20.2 ± 1.0		37.1 ± 0.2		20.4 ± 0.5	
Aw (3)	32.8 ± 0.7		17.0 ± 0.6		18.4 ± 1.9		36.0 ± 0.7		19.0 ± 1.1	
BS (6)	36.2 ± 0.3		17.0 ± 2.4		12.2 ± 2.3		40.2 ± 0.6		23.2 ± 2.1	
Cf (19)	33.3 ± 0.8		13.8 ± 0.9		16.4 ± 0.9		38.0 ± 0.5		24.1 ± 1.2	
Cs (14)	28.2 ± 0.8		15.8 ± 0.8		13.0 ± 0.8		34.2 ± 0.5		18.5 ± 1.1	
Cw (4)	34.9 ± 0.8		15.6 ± 1.1		15.3 ± 2.6		39.2 ± 0.8		23.6 ± 1.6	
Df (8)	30.8 ± 0.8		17.6 ± 1.4		16.1 ± 0.6		37.5 ± 0.6		19.9 ± 1.3	
	$F_{6,60} = 11.13$, $p = 0.002$		$F_{6,60} = 1.50$, $p = 0.648$		$F_{6,60} = 5.50$, $p = 0.04$		$F_{6,60} = 10.99$, $p = 0.001$		$F_{6,60} = 3.31$, $p = 0.206$	
Af - Aw	0.34	0.759	-0.12	0.995	0.72	0.563	0.90	0.419	0.50	0.908
Af - BS	-2.37	0.206	-0.18	0.995	**4.51**	**0.053**	**-3.60**	**0.025**	-1.39	0.628
Af - Cf	0.11	0.910	2.10	0.431	**2.76**	**0.053**	-1.35	0.294	-2.43	0.252
Af - Cs	5.23	0.046	0.62	0.995	4.88	0.088	3.96	0.135	1.16	0.908
Af - Cw	-1.10	0.314	0.51	0.995	2.29	0.088	-2.06	0.068	-1.34	0.396
Af - Df	2.26	0.251	-0.53	0.995	2.41	0.354	-0.54	0.780	0.23	0.908
Aw - BS	-1.96	0.113	-0.01	0.995	2.43	0.088	**-3.32**	**0.014**	-1.42	0.441
Aw - Cf	-0.29	0.785	1.37	0.679	0.89	0.504	-1.73	0.097	-1.96	0.252
Aw - Cs	2.89	0.113	0.51	0.995	2.29	0.363	1.54	0.428	0.21	0.908
Aw - Cw	-1.12	0.217	0.49	0.995	1.12	0.354	**-2.32**	**0.025**	-1.43	0.263
Aw - Df	1.20	0.404	-0.24	0.995	0.93	0.633	-1.22	0.428	-0.32	0.908
BS - Cf	2.65	0.113	1.94	0.740	-2.55	0.200	2.75	0.063	-0.53	0.908
BS - Cs	**6.88**	**0.011**	0.71	0.995	-0.48	0.837	**7.06**	**0.011**	2.41	0.396
BS - Cw	0.79	0.541	0.61	0.995	-1.31	0.418	0.83	0.494	-0.17	0.908
BS - Df	**4.18**	**0.016**	-0.30	0.995	-2.02	0.363	2.83	0.074	1.48	0.626
Cf - Cs	**5.73**	**0.014**	-1.50	0.995	2.57	0.504	**5.83**	**0.025**	3.83	0.396
Cf - Cw	-1.23	0.251	-0.87	0.995	0.55	0.669	-1.26	0.278	0.23	0.908
Cf - Df	2.35	0.113	-2.40	0.431	0.19	0.880	0.59	0.707	2.37	0.263
Cs - Cw	**-4.75**	**0.011**	0.09	0.995	-1.06	0.669	**-4.84**	**0.011**	-2.16	0.506
Cs - Df	-2.31	0.314	-1.09	0.995	-1.86	0.549	-4.07	0.057	-0.79	0.908
Cw - Df	2.72	0.081	-0.87	0.995	-0.36	0.837	1.54	0.364	1.43	0.582

Regarding the performance breadth, there were no significant differences between climatic regions but they did differ in the upper limit of B_{80}. Species from dry climates (B) showed higher B_{80max} than those from tropical (A) or temperate (C) regions. When considering a finer scale, dry semiarid species (BS) still had higher B_{80max} than the tropical groups (Aw and Af), however they only differed from the Mediterranean species (Cs) and not from the other temperate species.

Table 1.4. Phylogenetic ANOVAs and post-hoc results comparing critical thermal maximum and Warming Tolerance amongst Köppen-Geiger groups (first letter only), including mean ± SE for traits. Significant results are presented in bold.

koppen (N)	**CT_{max}**		**WT**		**WT_w**	
A (11)	40.4 ± 0.4		13.3 ± 0.8		11.0 ± 0.4	
B (6)	43.1 ± 0.4		5.4 ± 1.4		8.2 ± 1.0	
C (36)	39.7 ± 0.4		9.7 ± 0.8		11.2 ± 0.7	
D (8)	39.3 ± 0.4		12.2 ± 1.3		13.0 ± 0.3	
	$F_{3,57}$ = 6.12, p = 0.008		**$F_{3,57}$ = 4.80, p = 0.018**		$F_{3,57}$ = 2.40, p = 0.168	
Post-hoc	t	*p*	t	*p*	t	*p*
A - B	**-2.88**	**0.018**	**3.50**	**0.012**	1.67	0.232
A - C	0.96	0.529	**2.37**	**0.051**	-0.13	0.92
A - D	1.21	0.529	0.55	0.735	-1.29	0.512
B - C	**4.07**	**0.012**	**-2.17**	**0.051**	-2.02	0.15
B - D	**3.75**	**0.015**	**-2.82**	**0.051**	-2.68	0.15
C - D	0.59	0.691	-1.44	0.356	-1.42	0.42

Table 1.5. Phylogenetic ANOVAs and post-hoc results comparing critical thermal maximum and Warming Tolerance amongst Köppen-Geiger groups (first two letters), including mean ± SE for traits. Significant results are presented in bold.

koppen (N)	CT_{max}		WT		WT_w	
Af (12)	40.6 ± 0.5		12.5 ± 1.0		11.2 ± 0.4	
Aw (3)	39.8 ± 1.0		15.4 ± 0.9		10.4 ± 1.0	
BS (6)	43.1 ± 0.4		5.4 ± 1.4		8.2 ± 1.0	
Cf (19)	40.4 ± 0.5		9.3 ± 1.1		9.6 ± 0.7	
Cs (14)	38.0 ± 0.4		11.6 ± 1.3		13.9 ± 1.2	
Cw (4)	41.9 ± 0.5		5.2 ± 1.9		9.7 ± 0.9	
Df (8)	39.3 ± 0.4		12.2 ± 1.3		13.0 ± 0.3	
	$F_{6,54}$ = 11.13, *p* = 0.002		$F_{6,54}$ = 4.02, *p* = 0.086		**$F_{6,54}$ = 4.70, *p* = 0.033**	
Post-hoc	t	*p*	t	*p*	t	*p*
Af - Aw	0.77	0.418	-1.02	0.393	0.41	0.703
Af - BS	**-2.99**	**0.042**	**3.09**	**0.042**	1.92	0.343
Af - Cf	0.21	0.823	1.79	0.122	1.37	0.454
Af - Cs	3.65	0.088	0.48	0.923	-2.08	0.504
Af - Cw	-1.43	0.1803	**2.83**	**0.007**	0.89	0.504
Af - Df	1.64	0.382	0.15	0.923	-1.20	0.601
Aw - BS	**-3.02**	**0.008**	**3.33**	**0.007**	1.07	0.464
Aw - Cf	-0.70	0.475	**2.32**	**0.042**	0.48	0.681
Aw - Cs	1.74	0.382	1.41	0.529	-1.90	0.464
Aw - Cw	**-1.83**	**0.042**	**3.17**	**0.007**	0.34	0.703
Aw - Df	0.44	0.775	1.13	0.529	-1.30	0.522
BS - Cf	**3.63**	**0.007**	-1.95	0.164	-0.98	0.575
BS - Cs	**6.59**	**0.007**	-2.95	0.122	-3.99	0.105
BS - Cw	1.14	0.332	0.10	0.923	-0.76	0.575
BS - Df	**4.51**	**0.007**	-2.95	0.063	-3.03	0.147
Cf - Cs	4.31	0.065	-1.50	0.688	-4.20	0.247
Cf - Cw	-1.75	0.094	1.78	0.092	-0.06	0.953
Cf - Df	1.736	0.278	-1.60	0.344	-2.79	0.147
Cs - Cw	**-4.40**	**0.008**	2.66	0.179	2.58	0.343
Cs - Df	-1.83	0.382	-0.30	0.923	0.75	0.734
Cw - Df	**2.77**	**0.055**	-2.70	0.066	-1.87	0.454

Furthermore, species from humid subtropical climate with dry winters (Cw) and precipitation year round (Cf) also had higher B_{80max} than those from the Mediterranean (Cs). Some tropical species (Aw) had lower B_{80max} than those from humid subtropical climates with dry winters (Cw). Species from dry climates (B) had higher CT_{max} estimates than those from other climatic regions (Table 1.4). Dry semiarid species (BS) only did not differ in CT_{max} from humid subtropical climate with dry winters species (Cw) (Table 1.5). The later (Cw species) had higher CT_{max} than species from the Mediterranean (Cs), hot summer continental (Df) and tropical climates (Aw).

Vulnerability to global warming

Thermal Safety Margins (Annexe 1-F) decreased with performance breadth (B80) and increased with B_{80min} (Table 1.6). TSM was also related to CT_{max} when estimated with datalogger data and to optimum temperature when estimated with WorldClim data. Regarding the thermal environment, species exposed to lower average and minimum environmental temperatures had broader TSMs. Warming Tolerance was related to optimum temperature and both upper and lower limits of the performance breadth. Similarly to TSM, WT was particularly related to the thermal environment, decreasing with maximum and average temperatures and diel temperature fluctuation.

Thermal Safety Margins increased with latitude and altitude, while Warming Tolerance was only related to altitude when estimated with datalogger information. Using datalogger information, Thermal Safety Margins of species from the tropical climates (A) were significantly lower than those from the temperate regions (C) (Table 1.7).

Table 1.6. PGLS results for the relation between Thermal Safety Margins and Warming Tolerance (calculated with WorldClim and datalogger information) and the thermal physiology and environmental measurements. Significant results are presented in bold.

	TSM_d	**WT_d**	**TSM_w**	**WT_w**
latitude	**λ = 0.00, β = 0.23 ± 0.04** **t = 6.50, p < 0.001**	λ = 0.00, β = 0.09 ± 0.06 t = 1.45, p = 0.153	**λ = 0.81, β = 0.34 ± 0.04** **t = 8.38, p < 0.001**	λ = 0.83, β = 0.03 ± 0.05 t = 0.61, p = 0.541
altitude	**λ = 0.00, β = 3.03 ± 0.61** **t = 4.93, p < 0.001**	**λ = 0.00, β = 0.00 ± 0.00** **t = 2.42, p = 0.019**	**λ = 0.97, β = 2.47 ± 0.54** **t = 4.56, p < 0.001**	λ = 0.86, β = 0.00 ± 0.00 t = -0.60, p = 0.554
T_{opt}	λ = 0.59, β = 0.07 ± 0.16 t = 0.45, p = 0.653	**λ = 0.00, β = -0.63 ± 0.16** **t = -3.96, p < 0.001**	**λ = 0.95, β = 0.36 ± 0.18** **t = 2.04, p = 0.046**	**λ = 0.82, β = -0.37 ± 0.13** **t = -2.76, p < 0.008**
z_{max}	λ = 0.51, β = -0.17 ± 0.11 t = -1.61, p = 0.111	**λ = 0.39, β = 0.35 ± 0.14** **t = 2.40, p = 0.019**	λ = 0.93, β = -0.18 ± 0.11 t = -1.71, p = 0.093	λ = 0.85, β = 0.01 ± 0.09 t = 0.10, p = 0.923
B_{80}	**λ = 0.69, β = -0.32 ± 0.10** **t = -3.21, p = 0.002**	λ = 0.01, β = 0.01 ± 0.16 t = 0.04, p = 0.971	**λ = 0.96, β = -0.23 ± 0.08** **t = -2.76, p = 0.008**	λ = 0.88, β = 0.11 ± 0.09 t = 1.24, p = 0.218
B_{80max}	λ = 0.44, β = -0.16 ± 0.20 t = -0.82, p = 0.417	**λ = 0.00, β = -1.00 ± 0.20** **t = -4.91, p < 0.001**	λ = 0.93, β = 0.40 ± 0.22 t = 1.83, p = 0.072	**λ = 0.83, β = -0.45 ± 0.17** **t = -2.62, p < 0.011**
B_{80min}	**λ = 0.75, β = 0.25 ± 0.09** **t = 2.69, p = 0.009**	**λ = 0.00, β = -0.29 ± 0.12** **t = -2.36, p = 0.022**	**λ = 0.96, β = 0.25 ± 0.08** **t = 3.30, p = 0.002**	**λ = 0.88, β = -0.17 ± 0.08** **t = -2.24, p = 0.029**
CT_{max}	**λ = 0.00, β = -0.58 ± 0.22** **t = -2.63, p = 0.011**	**λ = 0.00, β = -1.27 ± 0.25** **t = -5.08, p < 0.001**	λ = 0.94, β = 0.07 ± 0.29 t = 0.23, p = 0.816	λ = 0.82, β = -0.31 ± 0.22 t = -1.38, p = 0.174
T_{max}	**λ = 0.00, β = -0.21 ± 0.07** **t = -3.05, p = 0.003**	**λ = 0.50, β = -0.79 ± 0.03** **t = -27.47, p < 0.001**	λ = 0.90, β = -0.18 ± 0.13 t = -1.40, p = 0.167	**λ = 0.80, β = -0.72 ± 0.05** **t = -14.87, p < 0.001**
T_{avg}	**λ = 0.84, β = -0.68 ± 0.07** **t = -10.22, p < 0.001**	**λ = 0.00, β = -0.46 ± 0.10** **t = -4.73, p < 0.001**	**λ = 0.93, β = -0.71 ± 0.07** **t = -10.05, p < 0.001**	**λ = 0.71, β = -0.31 ± 0.08** **t = -4.04, p < 0.001**
T_{min}	**λ = 0.00, β = -0.38 ± 0.04** **t = -8.92, p < 0.001**	λ = 0.00, β = -0.11 ± 0.09 t = -1.25, p = 0.215	**λ = 0.91, β = -0.47 ± 0.05** **t = -10.12, p < 0.001**	λ = 0.86, β = 0.01 ± 0.06 t = 0.13, p = 0.899
$DTF_{avg/annual}$	λ = 0.58, β = 0.08 ± 0.11 t = 0.74, p = 0.461	**λ = 0.00, β = -0.95 ± 0.11** **t = -8.48, p < 0.001**	**λ = 0.97, β = 0.48 ± 0.13** **t = 3.65, p <0.001**	**λ = 0.90, β = -0.54 ± 0.12** **t = -4.48, p < 0.001**

However, this difference was not evident when considering a finer scale (Table 1.8). Using WorldClim information, Thermal Safety Margins of species from the continental climates (D) were significantly higher than those from all other climates (Table 1.7). Furthermore, species from the tropical climates (A) also had lower Thermal Safety Margins than those from the temperate regions (C). When considering a finer scale, the observed patterns were very similar. In addition to the previous observations, dry semiarid species (BS) had lower thermal margins than Mediterranean species (Cs) (Table 1.8). Species from humid subtropical climate with dry winters (Cw) did not differ from the tropical climates (Aw and Af) while species from humid subtropical climate with precipitation year round (Cf) differed from Tropical rainforest species (Af).

Table 1.7. Phylogenetic ANOVAs and pots-hoc results for comparison of Thermal Safety Margins (calculated with datalogger and WorldClim environmental information) amongst Köppen-Geiger groups (first letter only), including mean ± SE of traits per group. Significant results are marked in bold.

koppen (N)	**TSM_d**		**TSM_w**	
A (15)	7.9 ± 0.5		9.0 ± 0.2	
B (7)	8.7 ± 0.3		11.1 ± 0.4	
C (37)	11.5 ± 0.7		13.7 ± 0.6	
D (8)	11.7 ± 0.9		19.6 ± 0.9	
	$F_{3,63} = 5.17$, $p = 0.034$		**$F_{3,63} = 21.03$, $p < 0.001$**	
Post-hoc	t	*p*	t	*p*
A - B	-0.58	0.816	-1.48	0.269
A - C	**-3.56**	**0.030**	**-4.83**	**0.002**
A - D	-2.60	0.244	**-7.67**	**0.002**
B - C	-1.99	0.228	-1.95	0.109
B - D	-1.69	0.332	**-5.18**	**0.002**
C - D	-0.13	0.938	**-4.81**	**0.002**

Table 1.8. Phylogenetic ANOVAs and pots-hoc results for comparison of Thermal Safety Margins (calculated with datalogger and WorldClim environmental information) amongst Köppen-Geiger groups (first two letters), including mean ± SE of traits per group. Significant results are marked in bold.

koppen	**TSM$_d$**		**TSM$_w$**	
Af (12)	7.4 ± 0.6		9.0 ± 0.3	
Aw (3)	9.5 ± 0.5		9.2 ± 0.7	
BS (7)	8.8 ± 0.3		11.4 ± 0.3	
Cf (19)	10.5 ± 0.8		12.3 ± 0.9	
Cs (14)	13.7 ± 1.1		16.3 ± 0.8	
Cw (4)	8.2 ± 0.6		11.1 ± 0.3	
Df (8)	11.7 ± 0.9		19.6 ± 0.9	
	$F_{6,60}$ = 5.57, *p* = 0.054		**$F_{6,60}$ = 16.84, *p* = 0.001**	
Post-hoc	t	*p*	t	*p*
Af - Aw	-1.07	0.464	-0.13	0.926
Af - BS	-0.90	0.655	-1.64	0.332
Af - Cf	-2.74	0.126	**-3.23**	**0.009**
Af - Cs	-5.22	0.126	**-6.68**	**0.004**
Af - Cw	-0.45	0.655	-1.33	0.227
Af - Df	-3.03	0.269	**-8.35**	**0.004**
Aw - BS	0.34	0.772	-1.01	0.403
Aw - Cf	-0.52	0.655	-1.79	0.082
Aw - Cs	-2.15	0.464	**-4.00**	**0.016**
Aw - Cw	0.56	0.624	-0.90	0.322
Aw - Df	-1.02	0.624	**-5.51**	**0.004**
BS - Cf	-1.32	0.597	-0.93	0.538
BS - Cs	-3.51	0.175	**-3.99**	**0.016**
BS - Cw	0.27	0.784	0.02	0.981
BS - Df	-1.84	0.464	**-5.85**	**0.004**
Cf - Cs	-2.96	0.464	-4.08	0.112
Cf - Cw	1.37	0.417	0.77	0.449
Cf - Df	-0.87	0.655	**-6.21**	**0.004**
Cs - Cw	3.16	0.269	3.28	0.074
Cs - Df	1.52	0.624	-2.67	0.227
Cw - Df	-1.83	0.464	**-4.97**	**0.004**

Differences in Warming Tolerance between climatic regions were only evident when using datalogger environmental information (Table 1.5). Species from dry climates (B) had lower Warming Tolerance than those from other climatic regions while tropical species (A) also had higher Warming Tolerance than those from temperate climates (C). However, these differences were less evident when considering a finer scale. Dry semiarid species (BS) and species from humid subtropical climate with dry winters have lower Warming Tolerance than those from tropical climates (Aw and Af). Furthermore, species from humid subtropical climate with precipitation year round (Cf) also differed from some tropical groups (Aw).

Species with lower Warming Tolerance (Annexe 1-F) also had narrower Thermal Safety Margins, either when calculating with datalogger ($\lambda = 0.00$, $\beta = 0.45 \pm 0.16$, $t = 2.82$, $p = 0.007$) or WorldClim ($\lambda = 0.79$, $\beta = 0.21 \pm 0.10$, $t = 2.14$, $p = 0.037$) environmental information. Estimates of Warming Tolerance and Thermal Safety Margins showed similar patterns, whether calculated using datalogger or WorldClim environmental temperature information (WT, Pearson's $r=0.80$, $p=0.005$; TSM, Pearson's $r=0.78$, $p=0.014$). Furthermore, estimates of WT and TSM tended to be higher when using WorldClim data, however this difference was only statistically significant for TSM ($t_{66}=7.13$, $p < 0.001$).

In several of the previous analysis, high values of lambda indicated that it was important to control for phylogeny. In addition, most of the thermal physiology traits also showed significant phylogenetic signal (Table 1.9).

Table 1.9. Phylogenetic signal of thermal physiology traits estimated using Pagel's λ, including optimum temperature (T_{opt}), maximum performance (z_{max}), performance breadth (B_{80}) and its upper (B_{80max}) and lower limits (B_{80min}). Significant results are marked in bold.

	Pagel's λ	***p*-value**
T_{opt}	**0.98**	**<0.001**
z_{max}	**0.81**	**<0.001**
B_{80}	0.00	1
B_{80max}	**0.97**	**<0.001**
B_{80min}	**0.57**	**0.01**

Discussion

In this work, we observed a trade-off between optimum temperature and performance breadth, where species with higher optimum temperature showing narrower performance breadths, and thus supporting "hotter is narrower". Nevertheless, as maximal performance is not inversely related to performance range, broadening of the thermal performance curve did not result in a loss of maximal performance. Hence, there is no empirical support in the thermal performance curves for generalist-specialist trade-offs, a result also reported in other works (e.g., Carrière and Boivin 1997, Palaima and Spitze 2004). Selection experiments have also provided mixed support for the importance of specialist-generalist trade-offs (Angilletta 2009), where selection for greater performance at one temperature does not always cause correlated decrease in performance at other temperatures (Bennett and Lenski 1993, Carrière and Boivin 2001, Anderson et al. 2005). Therefore, a “Jack-of-all-temperatures” does not have to be a master of none (Angilletta 2009). Although other works with ectothermic vertebrates show that “hotter is better” for locomotion performance (Van Berkum 1986, Bauwens et al. 1995, Wilson 2001), where taxa with higher optimum temperatures also perform better than taxa with lower optimum temperatures (Huey and Kingsolver, 1989; Savage et al., 2004), we could not find support for this hypothesis.

When comparing the thermal physiology traits with environmental measurements, most phylogenetic analysis which yielded significant results also show a high value of λ (Table 1.1). In addition, all thermal physiology traits, except for performance breadth B_{80}, presented significant high values of Pagel's λ (Table 1.9), which indicates that it is important to account for the phylogeny and that species may show little variation within closely related taxa (phylogenetic signal). Hence, their thermal characteristics could have resulted from evolutionary phylogenetic constraints (phylogenetic inertia) and therefore be evolutionarily conservative (Huey 1982, Rosen 1991, Kellermann et al. 2012). The presence of phylogenetic signal in physiology traits is consistent with previous works (Duarte et al. 2012, Kellermann et al. 2012, Gutierrez-Pesquera et al. unpublished data). Furthermore, most of the physiology traits measured, such as optimum temperature, CT_{max} and the upper and lower limits of B_{80}, were strongly related to environmental temperature, which might explain why species from different climatic regions varied in those traits in spite of sharing family lineages. Our results also agree with previous findings, showing optimum temperature and CT_{max} to be co-adaptive traits (Angilletta 2009, Huey et al. 2009).

Apart from the environmental thermal regimen, water availability (hydroperiod) also plays an indirect role on the thermal physiology, which is evident when comparing subtropical/temperate climates. Subtropical species are not seasonally limited by water availability (Cf, precipitation year round) and those species that are limited to breeding in the summer (Cw, dry winters) usually have higher optimum temperatures. However, in the Mediterranean basin (Cs), raining season occurs mainly in late autumn, winter and spring, which restricts the breeding season of Mediterranean amphibian species to the colder months of the year, as opposed to other subtropical/temperate groups. This might explain why Mediterranean early breeders are amongst those with lower optimum temperature and CT_{max}.

A strong dependence on the environment is also evident when estimating Warming Tolerance and Thermal Safety Margins. Although thermal physiology does have some effect on these estimates, most of the variation derives from differences in the environmental temperatures. Species with narrower Thermal Safety Margins are mainly from lower latitude regions, where average environmental temperature is higher during the breeding season, such as tropical (Af and Aw), semiarid (BS) and some subtropical climates (Cf). This relation with latitude was not observed in Warming Tolerance because tropical species are exposed to lower maximum environmental temperatures than those from mid-latitude climatic regions such as semiarid (BS) and subtropical climates (Cf and Cw). Furthermore, these analyses also showed that higher latitude species do not differ from tropical species and tend to have higher Warming Tolerance than those from intermediate latitudes. In addition, species living at higher altitudes show broader Thermal Safety Margins than their lowland counterparts and therefore seem less vulnerable to chronic negative effects of increasing environmental temperatures. Such pattern was not found for acute thermal stress (Warming Tolerance).

Previous studies of Thermal Safety Margins and Warming Tolerances found that the majority of species have positive warm safety margins, except for some tropical species or mid-latitude desert species (Deutsch et al. 2008, Huey et al. 2009, Clusella-Trullas et al. 2011, Diamond et al. 2012). Similar results were found in recent works with amphibian larvae, including the present study, where Thermal

Safety Margins and Warming Tolerances estimated using microhabitat data were mainly positive (Duarte et al. 2012, Gutierrez-Pesquera et al. unpublished data). However, Sunday et al. (2014) raised concern over the use of maximum air temperature in previous studies, demonstrating that it overestimates true Warming Tolerances and thermal-safety margins and underestimates the importance of behavioural thermoregulation to cope with both contemporary and future conditions. We also observe that the use of air temperature data from WorldClim (Hijmans et al. 2005) overestimates Warming Tolerance and Thermal Safety Margins measurements when compared to those estimated using microhabitat (datalogger) environmental information, although offering similar qualitative patterns (see Chapter 2). Furthermore, when calculating Thermal Safety Margins using maximum environmental temperature, we found that several amphibian species are already exposed to environmental temperatures that are very close or even surpass their optimum temperature, during their larval stage. These species are located not only in subtropical semiarid regions, where maximum environmental temperatures are higher, but also on other subtropical and temperate communities. Thermal performance curves of ectotherms are generally skewed towards colder temperatures, regardless of the climatic region, meaning that performance decreases much faster at temperatures above the optimum temperature than at temperatures below (Huey and Stevenson 1979, Huey and Kingsolver 1989, Frazier et al. 2006, Tewksbury et al. 2008). Hence, experiencing environmental temperatures above the optimum temperature can be dangerous and have negative consequences even if the organism's body temperature does not reach its critical thermal maximum. Therefore, although our main results indicate that in general species from lower latitudes (from particular climatic regions) are more vulnerable to global warming, species from higher latitudes can also be negatively affected by increasing environmental temperatures.

Amphibians, in particular in the adult stage, have several ways of coping with environmental temperature behaviourally, including aestivation (Wells 2007). Although tadpoles are also capable of some behaviour thermoregulation, their inability to switch habitats (without reaching metamorphosis) may restrict their access to cooler microclimates and limit their choice to the thermal environments existing in their aquatic habitats. Furthermore, the necessity of thermoregulation may result in narrower foraging windows or increased predation risk, thus affecting the growth and development of tadpoles. Species that breed in temporary or ephemeral habitats are also time constrained during this life-stage. Increasing environmental temperatures may result in shorter hydroperiod, since faster water evaporation would decrease pond duration, and although tadpoles do show some phenotypic plasticity in time to metamorphosis, the reduction in development time results in juveniles emerging smaller, with proportionately shorter limbs, and increased oxidative stress (Gomez-Mestre et al. 2013).

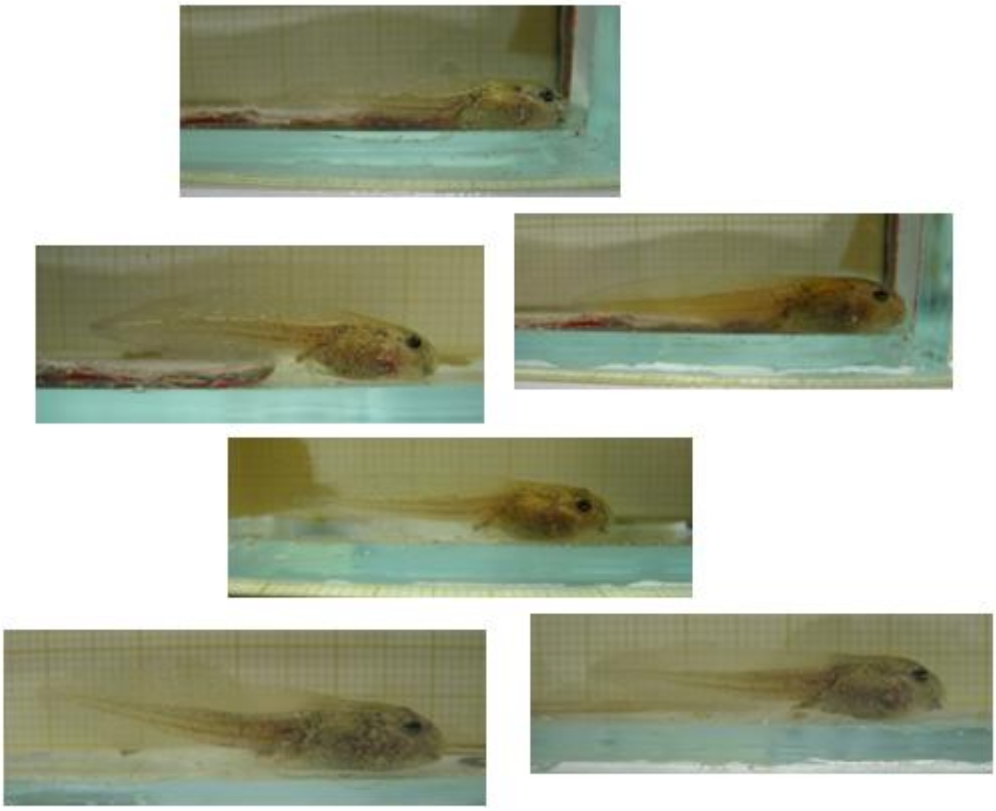
Conclusions

Determining the communities and species most vulnerable to climate warming is a challenging but important task (Somero 2010, Sinervo et al. 2010). According to our estimates of Warming Tolerance and Thermal Safety Margins, species from tropical and subtropical warm communities should be more vulnerable to increasing environmental temperatures, as seen in previous works (Deutsch et al. 2008, Huey et al. 2009, Duarte et al. 2012). However, as geographic variation in CT_{max} (Duarte et al. 2012, Grigg and Buckley 2013, Araújo et al. 2013, Sunday et al. 2014) and in physiologically optimal temperatures (this work; Huey et al. 2009) is limited, predictions of ectotherm vulnerability that are based only on the environmental temperatures of their present distributions (Foden et al. 2013) or comparisons of physiological limits to environmental temperatures (Deutsch et al. 2008, Sunday et al. 2012, Duarte et al. 2012), may miss the full story (Sunday et al. 2014).

Although we obtained positive Warming Tolerance and Thermal Safety Margins estimates, our data also shows that species, including some from higher latitudes, already experience temperatures very close or even above their optimal performance temperatures. To survive climate warming, ectotherms in most areas may need to rely on behaviours, and have access to habitats, that provide a reprieve from extreme operative temperatures (Sunday et al. 2014). However, species' ability to modify behaviours to thermoregulate (e.g., timing of activities) can have associated fitness costs, such as narrower foraging windows or increased predation risk, although these abilities and their costs are likely to vary with habitat (Huey and Slatkin 1976, Kearney et al. 2009b, Huey et al. 2009, Sunday et al. 2014).

Ultimately, organisms with a low tolerance for warming, limited acclimation ability, and reduced dispersal are more vulnerable to rapid climate change (Deutsch et al. 2008). In the current scenario of global warming, amphibians are presented with several challenges. As they present limited dispersal capability, in particular during their larval stage, amphibians will have to rely increasingly on behavioural thermoregulation and acclimation of their physiology to cope with rising environmental temperatures. Hence, future predictions of amphibian vulnerability to climate change should incorporate information on behavioural thermoregulation as well as estimates of the energetic consequences of extended thermal retreats (Sinervo et al. 2010, Huey et al. 2012, Sunday et al. 2014).

CHAPTER 2



Thermal physiology variation and vulnerability to thermal stress in Pelodytes ***spp. populations from the Iberian Peninsula***

Resumen

Predecir respuestas biológicas a cambios climáticos actuales enfatiza la necesidad de entender la fisiología térmica de las especies y de evaluar su capacidad de enfrentar estos cambios a través de su potencial de plasticidad fenotípica o evolutiva. En este trabajo estudiamos la variación en tolerancias térmicas máximas (CT_{max}) y en sensibilidad térmica (curvas de desempeño térmico, incluyendo la temperatura óptima) de poblaciones de dos especies diferentes de *Pelodytes* sp., para investigar si su fisiología térmica es filogenéticamente limitada o si hay una adaptación al ambiente térmico local. También evaluamos la susceptibilidad de cada población a los cambios térmicos agudos y crónicos mediante el cálculo de la Tolerancia al Calentamiento y de las Márgenes de Seguridad Térmica, respectivamente. Demostramos que *P. ibericus* y *P. puntactus* presentan fisiologías térmica similares a pesar de ser filogenéticamente distintas. Por lo tanto, la fisiología térmica de esas especies parece ser evolutivamente conservadora y la pequeña variación observada podría haberse obtenido y/o mantenido por la deriva genética. Como no encontramos evidencia de adaptación local, la fisiología térmica de las poblaciones de *Pelodytes* sp. puede estar reflejando la distribución de los linajes determinada por el ambiente. Aunque las poblaciones del género *Pelodytes* con temperaturas óptimas altas tienden a ser térmicamente más especializadas, indicando que "más caliente es más estrecho" (del inglés “hotter is narrower”), este resultado no fue observado al nivel de especie. Nuestros datos también sugieren que un "Juan-de-todas-las-temperaturas" (del inglés, “Jack-of-all-temperatures”) no tiene por qué ser un maestro de nada y que sólo hemos encontrado soporte a la hipótesis "más caliente es mejor" (del inglés, "hotter is better") en *P. puntactus*. Por otra parte, nuestras estimaciones de Tolerancia al Calentamiento y Márgenes de Seguridad Térmica indican una diferencia importante entre la fisiología térmica (CT_{max} y T_{opt}) y el ambiente térmico, apoyando la idea de que las especies templadas tienen cierto margen para hacer frente al estrés térmico ambiental.

Palabras clave: temperatura óptima, temperatura crítica máxima, curvas de desempeño térmico, variación intraespecífica, anfibios, comparaciones P_{st}-F_{st}.

Abstract

Forecasting biological responses to current climatic changes emphasizes the necessity of understanding species thermal physiology and to assess their potential to face these changes via either plasticity or evolution. We studied variation in upper thermal tolerances (CT_{max}) and thermal sensitivity (thermal performance curves, including optimum temperature) of 12 populations from two different *Pelodytes* sp. species, to investigate whether their thermal physiology is phylogenetic constrained or if there is adaptation to the local thermal environment. We also evaluated each population's susceptibility to acute and chronic thermal changes by calculating their Warming Tolerance and their Thermal Safety Margins, respectively. We demonstrated that *P. ibericus* and *P. puntactus* share similar thermal physiology characteristics despite being phylogenetically distinct. Therefore, the thermal physiology of these species appears to be evolutionarily conserved and the small variation observed could have been achieved and/or maintained by genetic drift. As we did not find evidence for local adaption, the thermal physiology of *Pelodytes* spp. populations could instead reflect environmental sorting of lineages. Although populations of genus *Pelodytes* with higher optimum temperatures tend to be more thermally specialized, indicating that "hotter is narrower", this result was not observable on a species level. Our data also suggest that a "Jack-of-all-temperatures" does not have to be a master of none; we only found support for "hotter is better" in *P. puntactus*. Furthermore, our estimates of Warming Tolerance and Thermal Safety Margins indicate an important difference between physiology traits (CT_{max} and T_{opt}) and the thermal environment, supporting the idea that temperate species have some margin to cope with environmental thermal stress.

Keywords: critical thermal maximum, optimum temperature, thermal performance curves, intraspecific variation, amphibians, P_{st}-F_{st} comparison

Introduction

In the current scenario of climate change, predictions by the IPCC for the coming decades include a five-fold increase in warming rate, an increase of extreme climatic events (Schär et al. 2004, Diffenbaugh and Ashfaq 2010), and alteration of global precipitation patterns (IPCC 2013). Forecasting biological responses to current climatic changes emphasizes the necessity of understanding how a species' physiological characteristics vary through space and time (Kearney and Porter 2009, Helmuth et al. 2010), and assess their potential to face these changes via either plasticity and/or evolution (Pörtner and Farrell 2008, Chown et al. 2010, Huey et al. 2012, Hoffmann et al. 2013). Hence, as temperature is an important factor responsible for environmental heterogeneity and since it affects virtually all physiological processes, such as determining rates of chemical reactions (Hochachka and Somero 2002) and many ecological interactions (Dunson and Travis 1991), renewed attention has been given to the study of thermal physiology of organisms, in particular their critical thermal limits and optimum temperature (e.g., Deutsch et al. 2008, Tewksbury et al. 2008, Huey et al. 2009, Duarte et al. 2012).

Closely related species may exhibit similar thermal characteristics (phylogenetic signal) due to evolutionary phylogenetic constraints (phylogenetic inertia). In this case, their thermal characteristics would be evolutionarily conservative, showing little variation within closely related taxa (Huey 1982, Rosen 1991, Kellermann et al. 2012). However, this similarity amongst related species may also result from spatial proximity. If so, closely related taxa sharing a common environment would have similar thermal needs, and share a similar thermal ecology, whereas populations in different environments would have different thermoregulatory mechanisms or the ability to tolerate suboptimal temperatures, but with no change in its ancestral physiological limits. Furthermore, common selection regimes, due to spatial proximity, may also result in species being similar (see Kellermann et al. 2012). Physiological adaptation hypothesis defines thermal characteristics to be evolutionarily labile, adapting themselves to the conditions of the local environment through selective pressure (Hertz et al. 1983). Hence, closely related taxa occupying different habitats may have their own optimal ranges and unrelated taxa sharing similar environments may evolve convergent thermal preferences with time.

In ectotherms, most physiological processes operate within the bounds of lethal temperature extremes and change rapidly over a range of body temperatures defining a thermal performance curve or TPC (Huey and Stevenson 1979). The performance of a physiological trait gradually increases with temperature from a minimum critical temperature (CT_{min}) to an optimum before dropping precipitously as temperature approaches a critical thermal maximum (CT_{max}). Temperatures either below or above the range of tolerance, defined by the critical thermal limits, result in impaired physiological function (Hillman et al. 2009). As animals often perform under sub-optimal environmental conditions (Huey et al. 1989, Martin and Huey 2008), there has been increasing interest not only on maximal performance capacity but also on the shape and position of the reaction norm describing the environmental dependence of physiological performance (Schlichting and Pigliucci 1998, Angilletta et al. 2003, Angilletta 2006, Kingsolver et al. 2007, Gvoždík and Van Damme 2008).

If adaptation is unable to overcome the rate-depressing effects of low temperature, organisms adapted to lower temperatures are predicted to have lower maximum performances (e.g., sprinting speeds and fitness) than those adapted to higher temperatures (Bennett 1987). In terms of continuous reaction norms, the “hotter is better” hypothesis would then predict a positive correlation between an organism’s optimal temperature and its maximum performance at the optimal temperature (Hamilton 1973, Huey and Kingsolver 1989, Savage et al. 2004, Frazier et al. 2006, Knies et al. 2009). In contrast, if organisms living at low temperatures can compensate for rate-depressing effects (Huey and Kingsolver 1989), then organisms inhabiting low temperatures should achieve the same maximum performances as those living at high temperatures (Frazier et al. 2006, Knies et al. 2009). Several comparative studies between species (or higher taxa) have shown strong support for this hypothesis (Eppley 1972, Bauwens et al. 1995, Rehfeldt et al. 2002, Heilmayer et al. 2004, Frazier et al. 2006; see Angilletta et al. 2010). However, support for “hotter is better” is quite mixed (or yields weaker results) for some aspects of performance, such as locomotion (Kingsolver 2009, Angilletta et al. 2010). Furthermore, if the “hotter is better” pattern that characterizes comparisons between species reflects limitations on adaptation of reaction norms, then intraspecific comparisons should also reflect those limitations and present that same pattern (Knies et al. 2009). Currently, experiments that studied the “hotter is better” hypothesis in an intraspecific context have produced mixed results, with some observing the pattern (e.g., Knies et al. 2009) and others not (e.g., Izem and Kingsolver 2005).

Another common assumption is that maximal performance or fitness is inversely related to temperature range, the ‘‘Jack- of-all- temperatures is a master of none’’ hypothesis (Huey and Hertz 1984). As a result of this thermal specialist-generalist trade-off, selection for an increased temperature range is expected to incur in a reduction in maximal performance and selection for maximal performance is expected to reduce an organism’s temperature range (Levins 1968, Huey and Slatkin 1976). If such trade-offs exist, then “hotter is better” can be achieved only through a narrowing of reaction norms, resulting in “hotter is narrower”. However, despite being a common assumption in evolutionary models of thermal adaptation (Lynch and Gabriel 1987, Gilchrist 1995), there is little empirical support for generalist-specialist trade-offs (Huey and Hertz 1984, Angilletta et al. 2002, Yamahira et al. 2007). If generalist-specialist trade-offs do not constrain adaptation to temperature, then hotter is better could generate a higher and broader reaction norm, increasing the temperature range (Knies et al. 2009).

Here we study variation in CT_{max} and thermal performance curves for burst swimming speed (including optimum temperature) of different amphibian populations of the genus *Pelodytes* and their thermal environment. We investigate whether closely related taxa, *Pelodytes ibericus* and *P. puntactus*, are phylogenetic constrained (phylogenetic inertia) and exhibit similar thermal characteristics or if these species’ populations are adapted to the conditions of the local environment (physiological adaptation hypothesis). Furthermore, we evaluate in an intraspecific context if our data supports the “hotter is better” hypothesis and if adaptation to temperature in burst swimming speed is constrained by a generalist-specialist trade-off. Finally, we also evaluate each population’s susceptibility to acute and chronic thermal changes (increasing environmental temperatures) by calculating their warming tolerance and their Thermal Safety Margins respectively (Deutsch et al. 2008).

Methods

For this work, we sampled tadpoles of 12 *Pelodytes* spp. populations from the Iberian Peninsula, comprising the two currently described species for this region, *P. puntactus* and *P. ibericus* (Table 2.1) (Sánchez-Herraíz et al. 2000). Field sampled larvae were then transported to the laboratory (EBD-CSIC, Sevilla, Spain). To assess the environmental temperatures to which these species are exposed during their breeding season, we considered two approaches: a) mapping a set of global climate layers (climate grids) available in WorldClim (Hijmans et al. 2005) and extracting temperature data for the collection sites using QGIS software; and b) recording water temperature every 5-15 min, using a HOBO Pendant® temperature datalogger placed in each collection site at the deepest part of the pond and only when it held water (during each population's springtime reproductive season). In both cases we extracted estimates of T_{avg} (average temperature), T_{max} (maximum temperature), T_{min} (minimum temperature), and DTF (diel temperature fluctuation; Table 2.1). We conducted ANOVA analysis to determine if species differed in their thermal environments (including altitude), testing each environmental measurement separately.

Table 2.1. Pond location, altitude and temperature measurements for 12 populations of *Pelodytes puntactus* and *P. ibericus*.

Population	Species	alt[¥]	latitude	longitude	datalogger				WorldClim			
					DTF_{avg}	T_{max}	T_{avg}	T_{min}	DTF_a	T_{max}	T_{avg}	T_{min}
São Luis	*P. puntactus*	76	37°43'05.16"N	08°42'47.75"W	-	-	-	-	9.1	17.4	12.3	7.8
Vila do Bispo	*P. puntactus*	126	37°06'59.57"N	08°53'34.06"W	2.3	22.0	16.5	12.8	8.4	16.9	12.4	8.3
Rocha da Pena/Penina	*P. puntactus*	350	37°15'08.01"N	08°05'07.54"W	-	-	-	-	9.6	16.3	10.8	5.9
Nave do Barão	*P. puntactus*	175	37°13'08.98"N	08° 2'58.33"W	1.4	18.7	13.3	10.7	9.5	17.4	11.8	6.8
Vale da Telha	*P. puntactus*	100	37°17'52.48"N	08°50'29.18"W	1.4	18.4	15.9	13.1	8.6	17	12.3	8
Jerez	*P. ibericus*	12	36°39'20.03"N	06°10'06.01"W	3.5	25.1	18.5	14.5	7.3	19.4	14.0	8.7
Trebujena	*P. ibericus*	14	36°51'31.85"N	06°11'17.77"W	5.8	27.9	18.5	10.6	8.2	19.6	13.7	7.9
Grazalema	*P. ibericus*	808	36°44'27.45"N	05°20'29.77"W	9.0	26.1	15.5	8.3	10.5	17.2	10.1	3.4
Cabra	*P. ibericus*	960	37°30'45.82"N	04°21'41.33"W	6.4	29.5	11.3	2.4	11.8	21.4	10.8	2.1
Toba	*P. ibericus*	585	37°59'39.88"N	04°54'07.82"W	8.0	26.6	15.9	8.2	12.3	19.8	11	3.2
Bicos/Colos	hybrid	105	37°48'03.03"N	08°33'02.40"W	4.7	20.3	15.6	10.7	9.5	17.3	12.0	7.5
Beas	*P. ibericus*	498	38°16'38.50"N	02°56'47.37"W	9.3	25.6	17.3	10.9	11.6	19.8	11.5	3.7

[¥] alt, altitude (m); DTF_{avg}, average diel temperature fluctuation (°C) for the breeding season; DTF_a, annual diel temperature fluctuation (°C); T_{max}, maximum environmental temperature (°C); T_{avg}, average environmental temperature (°C); T_{min}, minimum environmental temperature (°C). Note: At Bicos/Colos collection site, *P. puntactus* and *P. ibericus* are sympatric and share the same breeding season. Since we could not determine which species each tadpole belonged to, this population was considered a "hybrid"

Thermal physiology of **Pelodytes** *spp.*

For thermal physiology trials, tadpoles were randomly selected and held individually in plastic containers with 0.5 L of water. Tadpoles were then fed rabbit chow *ad libitum* and maintained at a constant room temperature of 20 °C, with photoperiod 12L:12D, for at least 4 d. We only tested tadpoles bellow 38 Gosner stage (Gosner 1960) since tadpoles above that stage have reduced thermal tolerances (Sherman 1980, Floyd 1983). CT_{max} estimates were obtained using Hutchison's dynamic method (Lutterschmidt and Hutchison 1997b). We exposed tadpoles to a constant heating rate of 1 °C/min, until they reached complete immobility, which we considered as the experimental endpoint (Lutterschmidt and Hutchison 1997b). Once CT_{max} was reached, we transferred each tested tadpole to water at room temperature (~20 °C) to allow for recovery, after which they were weighed. Each individual was tested only once and each test container had only one tadpole per trial. In total, 173 tadpoles were used in the CT_{max} trials (15 tadpoles per population with the exception of Beas population, N= 8, see Table 2.2). To examine if species and populations differed in CT_{max}, we performed a nested ANOVA analysis, with CT_{max} as dependent variable, and population nested within species. Tadpole mass was excluded from analysis as it was not related to CT_{max}. As Colos/Bicos tadpoles are either hybrids or a mix of tadpoles from the two studied species, we did not include this population in the analysis.

We estimated thermal sensitivity using thermal performance curves based on locomotor performance (burst swimming speed). Locomotor performance has been employed as a good proxy to estimate optimal temperatures in ectotherms since it may correlate with fitness (Jayne and Bennett 1990, Le Galliard et al. 2004, Husak 2006). To determine burst swimming speed (i.e. burst speed), tadpoles were placed individually on a portable thermal bath (patent license ES 2372085), which consists of an opened cross section methacrylate tube (1 m long x 6 cm wide x 3 cm deep) filled with water of a given temperature. We then gently prodded the specimen with a thin stick to stimulate swimming. Each trial was recorded using a digital camera (30 frames/s) positioned above the tube (JVC Everio GZ-MG505). TPCs were defined using a set of six temperatures (10°, 15°, 20°, 24°, 28° and 32°C) that were tested in a random order (same order for all populations). Prior to swimming, tadpoles were submitted for approximately an hour to the test temperature.

Table 2.2. Critical thermal maximum (CT_{max}) estimates for 12 *Pelodytes* sp. populations.

Population	n	CT_{max} (°C ± SE)	mass (mg ± SE)
São Luis	15	36.8 ± 0.1	302.9 ± 12.1
Vila do Bispo	15	36.9 ± 0.1	251.0 ± 20.5
Rocha da Pena/Penina	15	37.3 ± 0.1	398.8 ± 28.0
Nave do Barão	15	37.0 ± 0.1	305.3 ± 18.9
Vale da Telha	15	37.1 ± 0.1	326.6 ± 19.4
Jerez	15	36.9 ± 0.0	306.8 ± 35.1
Trebujena	15	37.1 ± 0.0	414.5 ± 23.4
Grazalema	15	37.2 ± 0.1	525.3 ± 59.1
Cabra	15	37.4 ± 0.1	242.5 ± 14.1
Toba	15	36.3 ± 0.1	311.8 ± 24.4
Bicos/Colos	15	36.8 ± 0.1	321.7 ± 16.6
Beas	8	37.0 ± 0.1	437.8 ± 52.6

We used Measurement in Motion v3.0 software (Learning in Motion 2004) to estimate burst speed over three frames (0.1 s) after the tadpole started to move by measuring the distance the centre of mass moved between frames (Arendt 2009, 2010). We then considered the fastest speed of three or more bouts as our final measure of burst speed. Since sprint speed may scale with body size (Gvoždík and Van Damme 2006) and body size may confound the effect of speed on escape success (Van Damme and Van Dooren 1999), we used size-corrected burst speed (tadpole total length) for constructing thermal performance curves (see Table 2.3). To describe the TPCs for burst speed, we used the Template Mode of Variation method (TMV; Izem and Kingsolver 2005) which employs a polynomial function to decompose variation among TPCs into three predetermined modes of variation with biological connotation: vertical shift (faster-slower), horizontal shift (hotter-colder), and specialist-generalist trade-offs (Huey and Kingsolver 1989; see Izem and Kingsolver 2005 for details on calculations).

Table 2.3. Total tadpole length (mean ± SE; TTL, from snout to tip of tail) and maximum burst swimming speeds (mean ± SE) at six experimental temperatures, for 12 populations of *Pelodytes* sp..

Population	n	TTL (mm)	Burst swimming speed (cm/s)					
			10 °C	**15 °C**	**20 °C**	**24 °C**	**28 °C**	**32 °C**
São Luis	27	27.3 ± 0.6	15.5 ± 0.8	22.7 ± 0.8	28.8 ± 1.3	30.0 ± 1.2	28.1 ± 0.8	26.0 ± 1.5
Vila do Bispo	22	28.7 ± 1.0	17.1 ± 0.9	21.3 ± 1.1	25.2 ± 1.5	28.1 ± 1.2	26.8 ± 1.3	23.9 ± 1.8
Rocha da Pena/Penina	53	33.7 ± 0.6	20.7 ± 0.7	24.8 ± 0.6	28.8 ± 0.9	32.3 ± 0.8	30.4 ± 0.8	28.0 ± 1.0
Nave do Barão	14	31.0 ± 1.3	17.7 ± 1.1	21.1 ± 1.7	23.9 ± 1.8	27.2 ± 1.7	25.8 ± 1.8	22.5 ± 2.0
Vale da Telha	9	29.6 ± 2.0	16.8 ± 2.1	22.4 ± 1.9	25.8 ± 1.8	31.8 ± 3.0	28.8 ± 2.6	25.7 ± 2.4
Jerez	25	28.7 ± 0.6	20.7 ± 0.9	25.4 ± 0.7	29.3 ± 1.2	31.4 ± 1.4	29.5 ± 1.7	26.7 ± 1.2
Trebujena	25	30.1 ± 0.5	18.0 ± 0.6	20.7 ± 0.5	23.3 ± 0.7	25.8 ± 0.8	24.6 ± 0.7	20.2 ± 1.0
Grazalema	25	29.7 ± 0.6	18.9 ± 0.7	24.6 ± 0.9	30.0 ± 1.0	30.4 ± 1.1	28.3 ± 0.9	25.4 ± 1.2
Cabra	26	37.6 ± 0.6	27.6 ± 0.9	32.5 ± 0.9	36.8 ± 1.3	42.4 ± 1.5	40.0 ± 1.6	37.3 ± 2.2
Toba	26	40.0 ± 0.5	29.9 ± 0.8	35.7 ± 1.0	40.5 ± 0.8	42.8 ± 1.3	42.1 ± 1.7	40.2 ± 2.7
Bicos/Colos	23	27.7 ± 0.9	19.2 ± 1.1	23.1 ± 1.0	26.7 ± 1.2	31.1 ± 1.5	28.7 ± 1.5	25.2 ± 1.4

Since we tested tadpole performance at six temperatures, we assumed that the common template curve was a fourth-degree polynomial, as in previous studies (Gvoždík and Van Damme 2008). We also included in the TPCs the overall average CT_{max} (~37°C), considering all individuals from all populations with performance 0 at that temperature, to get a better estimate of the common TPC shape (polynomial). For each population, we then used the common polynomial and swimming data (including performance 0 at the population's CT_{max}) with the *nlinfit* and *nlparci* functions in Matlab (The MathWorks 2013), to calculate new TPC parameters and respective standard error (SE). Furthermore, for each population we calculated maximum performance (z_{max}) and performance breadth B_{80}, (range of temperatures at which performance values exceed 80% of the maximum; Huey and Stevenson 1979).

We performed ANOVA analysis to determine whether these species differed in their physiological traits, testing each TPC parameter separately. We also used simple Pearson product-moment correlations to access the relation amongst the measured physiological traits (TPC parameters and CT_{max}) and with the measured environmental information (including altitude).

P_{st} - F_{st} comparisons for thermal physiology traits

In order to examine the thermal physiology data for indirect (phenotypic) signals of divergent selection, we compared the extent of divergence for the physiology traits, quantified as P_{st}, with neutral molecular divergence (F_{st}). P_{st} is analogous to Q_{st} (Raeymaekers et al. 2007), which is a measure of the genetic differentiation among populations for quantitative traits (Spitze 1993, see Whitlock 2008), and under divergent selection P_{st} will be larger than expected on the basis of neutral loci (Leinonen et al. 2006). P_{st} values for the physiological traits CT_{max}, T_{opt}, B_{80} and z_{max} were calculated as described in Raeymaekers et al. (2007). To determine the neutral genetic differentiation, we employed GenAlEx 6.5 (Peakall and Smouse 2012), on a dataset of 14 microsatellite markers available in GeneBank (Diaz-Rodriguez et al, unpublished data), to calculate F_{st} values (Weir and Cockerham 1984) and Nei's genetic distance values, for the studied *Pelodytes* populations. The comparison between P_{st} and F_{st} was interpreted as significantly different when the confidence intervals ($\alpha=0.05$) of the two divergence estimators did not overlap. We also calculated pairwise values for P_{st} and F_{st} for all population pairs and examined the relationship between the matrices of pairwise P_{st} and F_{st} values with a mantel test of matrix correspondence (Mantel 1967), as implemented in GenAlEx 6.5 (Peakall and Smouse 2012). In the P_{st} - F_{st} comparison for CT_{max}, Beas population was also included, hence a second F_{st} matrix was calculated for this analysis only.

Furthermore, we used Matlab to determined two matrices of pairwise Euclidean distances between populations; one including all physiological traits (CT_{max}, T_{opt}, B_{80}, B_{80max}, B_{80min} and z_{max}) and another with WorldClim environmental data (DTF, T_{avg}, T_{max} and T_{min}). Beas population was excluded from this analysis since we only had CT_{max} data available. We then compared both matrices with the pairwise Nei genetic distance and the geographic distance matrices, also using the Mantel test of matrix correspondence in GenAlEx 6.5. We controlled for the false discovery rate (FDR) in these Mantel tests using the Benjamini–Hochberg procedure (Benjamini and Hochberg 1995). Significance of all Mantel tests was obtained with 1000 permutations.

Vulnerability to increasing environmental temperatures assessment

For each population, we assessed its vulnerability to acute and chronic thermal changes, using two metrics defined in Deutsch et al. (2008): Warming Tolerance (WT), which is the difference between species' upper critical thermal limit (CT_{max}) and its current (maximum) environmental temperature (T_{max}); and Thermal Safety Margins (TSM), which is the difference between the species' thermal optimum (T_{opt}) and its current (mean) environmental temperature (T_{avg}). We used simple Pearson product-moment correlations to access the relation of these two metrics with the measured physiological traits (TPC parameters and CT_{max}) and environmental information (including altitude). We performed ANOVA analysis to determine whether these species differed in their vulnerability to thermal stress. We also compared WT and TSM estimates when using datalogger or WorldClim environmental information, by performing two-sample *t*-tests (WT and TSM separately). All analysis reported were performed in Matlab (The MathWorks 2013), except when mentioned otherwise, and were conducted on a significance level of α=0.05.

Results

Some differences were found between the two species in their thermal environments during their respective springtime breeding season. *Pelodytes puntactus* populations were exposed to lower maximum environmental temperature than *P. ibericus*, using both datalogger ($F_{(1,7)}$=33.1, p<0.001) and WorldClim $F_{(1,9)}$=15.9, p=0.003) information, and they also had lower average DTF (datalogger; $F_{(1,7)}$=15.8, p=0.005). However, these species did not differ in annual DTF (WorldClim) or in any of the other environmental temperature measurements taken, including altitude (all p>0.05).

Table 2.4. Parameters of thermal performance curves (mean ± SE) for burst swimming speed for 11 *Pelodytes* sp. populations, estimated with TMV method (Izem and Kingsolver, 2005) and nlinfit/nlparci functions in Matlab (Mathworks, 2013).

Population	**Thermal performance curve parameters**						
	w$^{¥}$	**T**$_{\mathbf{opt}}$	**h**	**z**$_{\mathbf{max}}$	**B**$_{\mathbf{80}}$	**B**$_{\mathbf{80max}}$	**B**$_{\mathbf{80min}}$
São Luis	1.19 ± 0.01	27.3 ± 0.1	0.12 ± 0.13	11.2	15.9	32.3	16.4
Vila do Bispo	1.28 ± 0.03	26.9 ± 0.2	-0.46 ± 0.17	9.8	16.4	32.2	15.8
Rocha da Pena/Penina	1.32 ± 0.02	27.0 ± 0.1	-0.40 ± 0.10	9.6	17.0	32.5	15.5
Nave do Barão	1.36 ± 0.05	26.6 ± 0.4	-0.97 ± 0.25	8.7	16.8	32.1	15.3
Vale da Telha	1.25 ± 0.03	27.3 ± 0.2	-0.35 ± 0.20	10.2	16.1	32.5	16.4
Jerez	1.28 ± 0.02	26.6 ± 0.2	0.64 ± 0.16	11.0	17.6	32.1	14.5
Trebujena	1.44 ± 0.03	26.0 ± 0.3	-0.68 ± 0.15	8.5	18.1	31.9	13.8
Grazalema	1.30 ± 0.02	26.8 ± 0.2	0.20 ± 0.14	10.4	17.4	32.3	14.9
Cabra	1.27 ± 0.02	27.0 ± 0.2	0.81 ± 0.16	11.2	17.8	32.6	14.8
Toba	1.23 ± 0.02	26.5 ± 0.2	0.63 ± 0.18	11.4	16.9	31.8	14.9
Bicos/Colos	1.25 ± 0.02	26.7 ± 0.2	0.43 ± 0.17	11.0	17.0	32.1	15.1

[¥] w, width (dimensionless); T_{opt}, optimal temperature (°C; m parameter in TMV method); h, height (TTL/s); z_{max} (TPC), maximum performance (TTL/s); B_{80}, thermal performance breadth at 80% (°C); B_{80max}, upper limit of B_{80} (°C); B_{80min}, lower limit of B_{80} (°C). Fourth degree polynomial, $-0.000454x^4-0.01096x^3-0.09427x^2+13.1844$.

Regarding the thermal physiology of genus *Pelodytes*, CT_{max} did not differ between species ($F_{(1,147)}=1.14$ $p=0.29$), however it did vary amongst populations ($F_{(9,147)}=20.4$ $p<0.001$), with the lowest value of 36.3 °C (± 0.1 SE) for the Toba population and the highest of 37.4 °C (± 0.1 SE) for the Cabra population. Tadpole mass was not correlated with CT_{max} estimates (Pearson's r=0.13, $p=0.13$) and therefore not included in the analysis.

Using the TMV method on the burst swimming speed data (see Table 2.3), we obtained a three-parameter shape-invariant model which explained over 84% of the variation for burst speed, with most of the variation being observed in the vertical (45.3%) and specialist-generalist (29.2%) directions, and less on the horizontal (10.1%). When studying the parameters of the thermal performance curves from the *Pelodytes* populations (Table 2.4), we observed that optimum temperature was negatively related to B_{80} (Pearson's r=-0.70, $p=0.017$) and both its upper and lower limits (B_{80max}, Pearson's r=0.84, $p=0.001$; B_{80min}, Pearson's r=0.87, $p<0.001$). In addition, B_{80} was related to B_{80min} (Pearson's r=-0.95, $p<0.001$) and CT_{max} varied with B_{80max} (Pearson's r=0.72, $p<0.012$). From all environmental measurements taken, only maximum environmental temperature (T_{max}) was related to both B_{80} and B_{80min}, either when using datalogger (B_{80}, Pearson's r=0.83, $p=0.011$; B_{80min}, Pearson's r=-0.78, $p=0.022$) or WorldClim information (B_{80}, Pearson's r=0.62, $p=0.04$; B_{80min}, Pearson's r=-0.62, $p=0.04$).

When considering the species separately, in *P. puntactus* populations the optimum temperature was related to maximum performance (Pearson's r=0.90, $p=0.038$). Also, *P. puntactus* had narrower performance breadths (B_{80}, $F_{(1,8)}=15.19$, $p=0.005$) and higher B_{80min} ($F_{(1,8)}=18.11$, $p=0.003$) than *P. ibericus*, but they did not differ in either maximum performance (z_{max}) or in B_{80} upper limit (B_{80max}). Furthermore, *P. puntactus* and *P. ibericus* populations had an average optimum temperature of 27.0 ± 0.2°C (mean ± SE) and 26.6 ± 0.2°C respectively, however this difference was marginally nonsignificant ($F_{(1,8)}=4.38$, $p=0.069$).

P_{st} - F_{st} comparisons for thermal physiology traits

Neutral molecular divergence, estimated as global F_{st}, was 0.151 (95% C.I 0.129-0.174) and the pairwise F_{st} values ranged between 0.031 (Nave do Barão - São Luis, Annexe II-A) and 0.324 (Colos/Bicos - Toba; Annexe II-A). With the inclusion of Beas population (for the CT_{max} P_{st} - F_{st} comparison only), global F_{st}

changed to 0.160 (95% C.I 0.148-0.172) and the pairwise F_{st} values ranged between 0.031 and 0.324 (Beas - Toba; Annexe II-A). Regarding the divergence of physiological traits in our dataset, we estimated global P_{st} to be: 0.21 (95% C.I 0.163-0.257; Annexe II-A) for CT_{max}; 0.098 (95% C.I 0.066-0.130; S2) for T_{opt}; 0.11 (95% C.I 0.078-0.142; S4) for B_{80}; and 0.201 (95% C.I 0.158-0.244; Annexe II-A) for z_{max}. Moreover, we observed that the confidence interval of the global F_{st} value overlapped with the confidence intervals of the global P_{st} values in all comparisons (including CT_{max}) and all the Mantel tests held between P_{st} and F_{st} matrices were nonsignificant (all $p>0.05$). However, when we consider all thermal physiology data simultaneously, the physiological distance matrix was correlated with Nei's genetic distance matrix ($R_{xy}=0.31$, $p=0.013$, Annexe II-B) but not with the geographic or environmental distance matrices (both $p>0.05$, Annexe II-B). In addition, Nei's genetic distance matrix was correlated with the geographic distance matrix ($R_{xy}=0.87$, $p=0.001$) and the environmental distance matrix ($R_{xy}=0.45$, $p=0.007$). Geographic distance and environmental distance matrices were also correlated ($R_{xy}=0.69$, $p=0.001$).

Warming Tolerance and Thermal Safety Margins

For the studied populations, we observed that our estimates of Warming Tolerance (Table 2.5) decreased with increasing DTF_{avg} (Pearson's r=-0.77, $p=0.015$) and T_{max} (datalogger, Pearson's r=-0.99, $p<0.001$; WorldClim, Pearson's r=-0.99, $p<0.001$). In addition, populations with higher warming tolerance had lower B_{80} (datalogger, Pearson's r=-0.81, $p=0.015$) and B_{80min} (datalogger, Pearson's r=0.78, $p=0.021$). We also observed that the populations' Thermal Safety Margins (Table 2.5) increased with altitude (WorldClim, Pearson's r=0.74, $p=0.009$; datalogger, Pearson's r=0.65, $p=0.059$) and annual DTF (WorldClim, Pearson's r=0.73, $p=0.011$) and, decreased with T_{avg} (datalogger, Pearson's r=-0.99, $p<0.001$; WorldClim, Pearson's r=-0.97, $p<0.001$) and T_{min} (datalogger, Pearson's r=-0.70, $p=0.037$; WorldClim, Pearson's r=-0.71, $p=0.014$).

When assessing both species vulnerability to increasing environmental temperatures, *P. ibericus* had lower Warming Tolerance than *P. puntactus* (datalogger, $F_{(1,7)}=35.3$, $p<0.001$; WorldClim, $F_{(1,9)}=14.\ 6$, $p=0.004$). However, they did not differ in their Thermal Safety Margins, using either datalogger or WorldClim data. Also, WT estimates were higher than TSM estimates when calculated using

WorldClim environmental data (t_{20}=6.05, $p<0.001$). Furthermore, our estimates of Warming Tolerance and Thermal Safety Margins showed similar patterns, whether calculated using datalogger or WorldClim environmental temperature information (WT, Pearson's r=0.80, p=0.005; TSM, Pearson's r=0.78, p=0.014), but they differed in the magnitude of the estimates (WT, t_{18}=4.1, $p<0.001$; TSM, t_{16}=3.8, p=0.002), with WT and TSM values being higher when using WorldClim data.

Table 2.5. Warming Tolerance (WT) and Thermal Safety Margins (TSM) for the studied populations of *Pelodytes* sp., calculated using either datalogger or WorldClim environmental information.

Population	**datalogger**		**WorldClim**	
	WT (°C)	**TSM (°C)**	**WT (°C)**	**TSM (°C)**
São Luis	-		19.4	15.0
Vila do Bispo	14.9	10.4	20.0	14.5
Rocha da Pena/Penina	-		21.0	16.2
Nave do Barão	18.3	13.3	19.6	14.9
Vale da Telha	18.7	11.4	20.1	15.1
Jerez	11.8	8.1	17.5	12.6
Trebujena	9.2	7.5	17.5	12.3
Grazalema	11.1	11.3	20.0	16.7
Cabra	7.9	15.7	16.0	16.2
Toba	9.7	10.6	16.5	15.5
Bicos/Colos	16.5	11.1	19.5	14.7
Beas	11.4	-	17.2	-

Discussion

Even though there is some variability in the environments to which each sampled population is exposed, the thermal habitats of *P. ibericus* and *P. puntactus* are quite similar, with these species not differing in minimum and average environmental temperatures and annual DTF. The differences found on maximum environmental temperature and average DTF can be attributed to the duration of the springtime breeding season. Tadpoles from the sampled Portuguese populations of *P. puntactus* are usually present until March, with higher incidence in February (Ferrand et al. 2001), while *P. ibericus* tadpoles can still be found as late as May, depending on the population (Díaz-Paniagua 1992, Barbadillo et al. 1999).

Although both *Pelodytes* species had similar average CT_{max}, we did find intraspecific CT_{max} variation, with differences between populations up to 0.5 °C in *P. puntactus* and 1.1 °C in *P. ibericus*. Moreover, if we disregard Toba population (which had the lowest CT_{max}), the remaining *P. ibericus* populations have a variation of only 0.5 °C, which is similar to *P. puntactus*. Recent works have reported that CT_{max} may differ adaptively between populations (e.g., Skelly and Freidenburg 2000, Wu and Kam 2005), in particular with altitude (Hertz 1979, Hertz et al. 1979), but not in others (Richter-Boix et al. (unpublished data). However, we found no relation between CT_{max} and altitude (as in other studies; e.g. Huey and Webster 1976, Gvoždík and Castilla 2001, Huang and Tu 2008) or the thermal environment.

Similarly to CT_{max}, there is some variation in optimum temperature both in *P. puntactus* (0.7 °C) and *P. ibericus* (1.0 °C) populations, although these were minor differences when compared to the amount of total variation attributed to changes in breadth and height of the thermal performance curves. We observed that *Pelodytes* populations with higher optimum temperature having narrower performance breadths (“hotter is narrower”). Nevertheless, although we observed “hotter is narrower”, changes in the generalist-specialist direction appear to have been compensated by vertical shifts (faster-slower), so that a broadening of the thermal performance curve did not result in a loss of maximal performance. As such, since maximal performance is not inversely related to temperature range, there is no empirical support in the thermal performance curves parameters for generalist-specialist trade-offs, a similar result to those reported in other works (e.g., Carrière and Boivin 1997, Palaima and Spitze 2004). Selection experiments have also provided mixed support for the importance of specialist-generalist tradeoffs (Angilletta 2009), where selection for greater performance at one temperature does not always cause correlated decrease in performance at other temperatures (Bennett and Lenski 1993, Carrière and Boivin 2001, Anderson et al. 2005). Hence, a “Jack-of-all-temperatures” does not have to be a master of none (Angilletta 2009).

Although other works with ectothermic vertebrates show that “hotter is better” for locomotion performance (Van Berkum 1986, Bauwens et al. 1995, Wilson 2001), we did not find support for this hypothesis, otherwise we would have observed populations with higher optimal temperatures also having higher burst swimming speed than populations with lower optimal temperatures (Huey and Kingsolver 1989, Savage et al. 2004). However, when considering each of our two species separately, we did find support for the “hotter is better” hypothesis in *P. puntactus* populations but not in *P. ibericus*, again showing mixed results for locomotion (see Kingsolver 2009, Angilletta et al. 2010). Also, we found no support for “hotter is narrower”, as we had found for genus *Pelodytes*, in either of the species (separately). Interspecific differences in B_{80min} (but not in B_{80max}) resulted in *P. ibericus* tadpoles having broader performance breadth towards colder temperatures, making them more generalist than *P. puntactus* tadpoles. Moreover, in *Pelodytes* sp. populations, the upper limit of the performance breadth (B_{80max}) appears to be constrained by CT_{max}.

In Bicos/Colos population, where *P. puntactus* and *P. ibericus* occur in sympatry, tadpoles presented similar physiological traits to both species. In particular, tadpoles from Bicos/Colos presented intermediate values for traits were differences between species were found (B_{80} and B_{80min}). This raises the question whether the physiological results obtained for this population are a result of tadpoles from both species being pooled together when sampling or if this population has hybrids of the two species that present an intermediate phenotype.

We found a significant signal of population differentiation across the *Pelodytes* spp. populations (F_{st} = 0.151; with Beas, F_{st} = 0.16). More specifically, *P. ibericus* populations were generally more genetically differentiated from those of *P. puntactus* than from other *P. ibericus* populations. The comparison of the patterns of genetic differentiation with the patterns of phenotypic differentiation (for each physiological trait separately) revealed no association between pairwise P_{st} and F_{st} values. Still, since global P_{st} and F_{st} values were similar for all comparisons, we infer that trait divergence among populations could have been achieved by genetic drift alone

(Leinonen et al. 2013). The use of microsatellites markers to determine patterns of genetic differentiation has been reported to give lower estimates F_{st}, thus upwardly biasing comparisons of P_{st} (Q_{st}) vs. F_{st} (Edelaar et al. 2011). Furthermore, some works have added estimates of trait heritability and additive genetic proportion of differences between populations to the calculation of P_{st}, showing that lower heritability values can also give higher P_{st} values (see Lehtonen et al. 2009). Therefore, one should keep in mind these potential biases when interpreting P_{st}-F_{st} comparisons.

When we consider each population's physiology as a whole (instead of each physiological trait separately), greater populational divergence exists as larger genetically distinction is found between populations. Again, this is consistent with genetic drift having some role in maintaining the observed patterns of phenotypic differentiation across *Pelodytes* spp. populations. In addition, as there was no correlation between the individual physiology traits and the environmental measurements or between the physiological distance matrix and the environmental distance matrix, we could not establish if these populations are currently under (strong) selective pressure from their thermal environment. Considering that these closely related taxa/populations show little variation in thermal physiology and since existing variation could have been achieved by genetic drift, these thermal characteristics may be under evolutionary phylogenetic constraints (phylogenetic inertia), making them evolutionarily conserved (Huey 1982, Rosen 1991, Kellermann et al. 2012). A previous work with *Drosophila* species found that current species distributions are more likely to reflect environmental sorting of lineages rather than local adaptation (Kellermann et al. 2012), which could be the case for *Pelodytes* spp. populations (or species).

Our assessment of the vulnerability of these populations to increasing environmental temperatures indicates that both species have similar Thermal Safety Margins but *P. ibericus* has lower Warming Tolerance than *P. puntactus*. Since their thermal physiology does not differ much, the thermal environment has a strong influence on these two metrics (WT and TSM). As *P. ibericus*' breeding season usually last longer than *P. puntactus* (Portuguese populations sampled), its populations are exposed to warmer days and higher DTF_{avg} (as spring progresses), thus resulting in different Warming Tolerances. Furthermore, populations from higher altitudes are less susceptible to suffer chronic thermal stress than lower altitude populations, as they are exposed to lower average environmental temperatures. However, we do not observe this pattern for Warming Tolerance, so that altitude does not influence susceptibility to acute thermal stress, at least for the studied populations.

In general, if we consider an equal increase in average and maximum environmental temperatures, these *Pelodytes* sp. populations should be more susceptible to chronic (rather than acute) thermal stress, as WT estimates were higher than TSM. However, current predictions also include an increase of extreme climatic events (Schär et al. 2004, Diffenbaugh and Ashfaq 2010). If events such as heat waves occur, a higher increase in maximum environmental temperature could reveal a higher (or equal) risk of acute thermal stress. Even so, the magnitude of our WT and TSM estimates reveals that *Pelodytes* sp. populations, like other temperate species (Deutsch et al. 2008, Duarte et al. 2012), still have some margin to cope with increasing environmental temperatures if other aspects of habitat suitability (such as rainfall patterns) remain appropriate.

Finally, the decision to use WorldClim or datalogger environmental information requires some attention, in particular for specific cases such as ephemeral or temporary aquatic habitats. WorldClim data is an interpolation of compiled monthly averages of climate for 1950–2000 (Hijmans et al. 2005), which eliminates a possible bias effect of an odd year, while the dataloggers used only measured one or two breeding seasons. However, dataloggers measured water temperature (the tadpoles' environment) instead of air temperature (WorldClim), giving a more precise measurement of the thermal microhabitat of the tadpoles. Our results indicate that warming tolerance and Thermal Safety Margins were consistently lower when estimated using datalogger information. This can reflect that either current the environmental thermal condition is hotter than the period considered in WorldClim or that the breeding seasons in which we measured water temperature with the dataloggers were abnormal. Nevertheless, estimating warming tolerance and Thermal Safety Margins using WorldClim data offered the same qualitative information as using datalogger measurements.

Conclusions

In this work, we demonstrated that *P. ibericus* and *P. puntactus* share similar thermal physiology characteristics despite being phylogenetically distinct (yet, closely related). We submit that phylogenetic inertia could have played a role in making the thermal physiology of these species (populations) evolutionarily conservative (Huey 1982, Rosen 1991, Kellermann et al. 2012). In addition, the little variation in thermal physiology could have been achieved, or at least maintained by genetic drift. As our estimates of warming tolerance and Thermal Safety Margins indicate an important difference between physiology traits (CT_{max} and T_{opt}) and the thermal environment, we could not establish if environmental temperatures are acting as a selective force and thus we found no evidence for local temperature adaption of populations. This indicates that the thermal physiology of *Pelodytes* spp. populations could reflect environmental sorting of lineages rather than local adaptation (see Kellermann et al. 2012). In addition, as these populations have relatively high Warming Tolerance and broad Thermal Safety Margins, it may also indicate that the current thermal environment does not impose a strong selection on their thermal physiology.

Populations of genus *Pelodytes* with higher optimum temperatures tend to be more thermally specialized (for burst swimming speed), indicating that “hotter is narrower”, but this result was not observable on a species level. Previous works had found that hotter can also be broader (e.g., Knies et al. 2009), which is also not the case. Furthermore, in one of the species (*P. puntactus*), we found support for “hotter is better”, but not in genus *Pelodytes* or in *P. ibericus* populations. Our data also suggests that a “Jack- of-all- temperatures” does not have to be a master of none, agreeing with previous works (see Angilletta 2009).

Current literature suggests that temperate species can be less susceptible to increasing environmental traits than some of their tropical counterparts (e.g. Deutsch et al. 2008, Duarte et al. 2012). Our effort corroborates the idea that temperate species (or at least the two tested here) have high Warming Tolerances and broad Thermal Safety Margins, even on a population level. Although WorldClim data facilitated acquisition of environmental temperature information (Hijmans et al. 2005), which is important specially for areas where access is limited, we still recommend placing dataloggers (whenever possible) in selected locations to better study the species' microhabitat (see Suggitt et al. 2011, Graae et al. 2012, Navas et al. 2013).

This would corroborate or complement the assessment of species' vulnerability to increasing environmental temperatures, in particular using WT and TSM metrics (Deutsch et al. 2008).

With some of the hypotheses we tested in this work yielding mixed results in several works, further research is needed to better understand the evolution and plasticity of thermal physiology and its constraints. For example, a recent work by Higgins et al. (2014) suggests that recent climate warming can lead to physiological shifts in TPCs, indicating that thermal adaptation can occur rapidly in response to changing thermal conditions. Therefore, this knowledge could also elucidate potential mechanisms for coping with current climate changes and improve physiologically based species distribution models (Buckley 2008, Kearney and Porter 2009, Kearney et al. 2009a, Overgaard et al. 2014) allowing for better predictions on the impacts on biodiversity (Pörtner and Farrell 2008, Chown et al. 2010).

CHAPTER 3

Swimming with predators and pesticides: How environmental stressors affect the thermal physiology of tadpoles

Resumen

Para predecir las respuestas biológicas frente a los cambios ambientales, necesitamos entender cómo la fisiología de una especie varía a través del espacio y del tiempo y evaluar cómo cambios en la función fisiológica debidos a cambios ambientales pueden interactuar con cambios fenotípicos causados por otros tipos de variación ambiental. Las larvas de anfibios son conocidas por expresar fenotipos inducidos por factores ambientales, pero se sabe poco sobre cómo esas respuestas pueden interactuar con los cambios de temperatura y con su fisiología térmica. Para abordar esa cuestión, estudiamos la fisiología térmica de renacuajos de rana arbórea gris (*Hyla versicolor*) determinando si la exposición a las señales de depredador y un herbicida (Roundup®) pueden alterar su temperatura crítica máxima (CT_{max}) y su velocidad de natación a través de un rango de temperaturas, que proporcionan estimaciones de la temperatura óptima (T_{opt}) para la velocidad de natación y la forma de la curva de desempeño térmico (TPC). Constatamos que las señales de depredadores indujeron un aumento de 0.4°C en el CT_{max}, mientras que el herbicida no tuvo ningún efecto. Los renacuajos expuestos a las señales de los depredadores o al herbicida, nadaron más rápido que los renacuajos del grupo control y ese aumento en la velocidad de natación fue mayor cerca de T_{opt}. Respecto a la forma de la TPC, la exposición a las señales de depredadores incrementó 1,5 ° C la T_{opt}, mientras que la exposición al herbicida bajó marginalmente la T_{opt} en 0,4° C. Combinando las señales de depredadores y el herbicida, se produjo un aumento de la T_{opt} de 0,5 ° C en comparación con el grupo de control. Según nuestro conocimiento, este es el primer estudio que demuestra un depredador alterando la fisiología térmica de larvas de anfibios (presa) aumentando el CT_{max}, aumentando la temperatura óptima, y produciendo cambios en las curvas de rendimiento térmico. Por otra parte, estas respuestas plásticas del CT_{max} y de la TPC a diferentes ambientes deben de ser consideradas al pronosticar las respuestas biológicas al calentamiento global.

Palabras clave: temperatura óptima, curvas de desempeño térmico, temperatura crítica máxima, plasticidad fenotípica, morfología inducida por depredadores, herbicida.

Abstract

To forecast biological responses to changing environments, we need to understand how a species' physiology varies through space and time and assess how changes in physiological function due environmental changes may interact with phenotypic changes caused by other types of environmental variation. Amphibian larvae are well known for expressing environmentally induced phenotypes, but relatively little is known about how these responses might interact with changing temperatures and their thermal physiology. To address this question, we studied the thermal physiology of grey treefrog tadpoles (*Hyla versicolor*) by determining whether exposures to predator cues and an herbicide (Roundup®) can alter their critical maximum temperature (CT_{max}) and their swimming speed across a range of temperatures, which provides estimates of optimal temperature (T_{opt}) for swimming speed and the shape of the thermal performance curve (TPC). We discovered that predator cues induced a 0.4°C higher CT_{max} value whereas the herbicide had no effect. Tadpoles exposed to predator cues or the herbicide swam faster than control tadpoles and the increase in burst speed was higher near T_{opt}. In regard to the shape of the TPC, exposure to predator cues increased T_{opt} by 1.5°C while exposure to the herbicide marginally lowered T_{opt} by 0.4°C. Combining predator cues and the herbicide produced an intermediate T_{opt} that was 0.5°C higher than the control. To our knowledge this is the first study to demonstrate a predator altering the thermal physiology of amphibian larvae (prey) by increasing CT_{max}, increasing the optimum temperature, and producing changes in the thermal performance curves. Furthermore, these plastic responses of CT_{max} and TPC to different inducing environments should be considered when forecasting biological responses to global warming.

Keywords: critical thermal maximum (CT_{max}), optimum temperature, thermal performance curves, predator-induced morphology, phenotypic plasticity, herbicide.

Introduction

Biological mechanisms underlying a response to environmental changes can be quite complex. To forecast these biological responses, we need to understand how a species' physiology varies through space and time (Kearney and Porter 2009, Helmuth et al. 2010) and assess how changes in physiological function induced by environmental changes (e.g., increasing environmental temperatures) may interact with phenotypic changes induced by other types of environmental variation (Chown and Terblanche 2007, Pörtner and Farrell 2008, Hoffmann 2010, Chown et al. 2010).

Species can possess the ability to respond to new or altered environments with flexible phenotypes that are environmentally induced and can potentially contribute to adaptive evolution (Ghalambor et al. 2007). Stressful environments can induce non-adaptive plasticity, increasing the variance around the mean phenotypic response or distancing it from the favoured optimum. Nevertheless, if plasticity is adaptive and promotes establishment and persistence in a new environment, by placing populations close enough to a new phenotypic optimum for directional selection to act, it can predictably enhance fitness and is most likely to facilitate adaptive evolution on ecological timescales (Ghalambor et al. 2007).

The presence of predators in the environment can induce behavioural and morphological changes in prey that result in the prey being less susceptible to the predator (e.g., Arnqvist and Johansson 1998, Relyea 2002, Domenici et al. 2008, Brönmark et al. 2011). Furthermore, pesticides can also induce behavioural and morphological changes in organisms. Sublethal exposure to pesticides early in life can make the individuals more tolerant of the pesticide later in life (Poupardin et al. 2008, Hua et al. 2013) and they can induce phenotypic changes that resemble predator-induced phenotypes (Hanazato 1991, Barry 1998, Oda et al. 2011, Relyea 2012). In other cases, pesticides impede the induction of predator-induced morphology (Barry 1999, 2000, Hanazato 1999, Sakamoto et al. 2006).

In the current scenario of climate change, there has been a renewed interest in the thermal physiology of organisms and the estimation of thermal tolerance and sensitivity, using physiological traits such as the critical thermal maximum (CT_{max}; e.g., the temperature at which animals become immobile [Hutchison 1961, Lutterschmidt and Hutchison 1997b]), the optimum temperature (T_{opt}) for performing some function, or the shape of the thermal performance curve (TPC), which describes how an animal's performance changes across a range of temperatures. Although some pesticides are known to affect CT_{max} and burst speed, usually in a negative way (e.g., Heath et al. 1997), there is limited information on how pesticides affect optimum temperature and performance over a range of temperatures (i.e. how pesticides affect TPCs), especially for amphibians. Likewise, much is known about predator-induced changes in organisms, including some interactions with pesticides (Relyea 2012). Predators also influence thermoregulation and thermal preferences of prey, resulting in behavioural changes and coevolution of thermal optima between species (Angilletta 2009). Other than these behavioural responses that indirectly affect physiology, little is known about whether predator cues can directly affect the thermal physiology of prey.

We addressed these issues by studying the thermal physiology of grey treefrog tadpoles (*Hyla versicolor* LeConte 1825) that were exposed to predator cues and pesticides. Tadpoles are excellent model organisms for this study because they are practically isothermal with their aquatic environment (Lutterschmidt and Hutchison 1997b) and their thermal physiology traits (CT_{max} and T_{opt}) are not influenced by confounding processes such as dehydration. Tadpoles are also well known for expressing predator-induced changes in behaviour and morphology (e.g., Relyea 2002, Van Buskirk 2002, Miner et al. 2005). Furthermore, at least two species of tadpoles can alter their morphology when exposed to the herbicide Roundup and exhibit morphological changes that closely resemble predator-induced changes in tadpoles (Relyea 2012).

Given that pollutants and predators can both affect many aspects of tadpole biology, including development and metamorphosis (e.g., Relyea 2007, Hayes et al. 2010), and the interaction of pollutants with other stressors are often negative to the organism (e.g., glyphosate, Wagner et al. 2013), we expect the impact of these stressors on the thermal physiology of tadpoles to be mainly negative. Therefore, we hypothesized that tadpoles exposed a sublethal concentration of an herbicide will have reduced tolerance to higher temperatures (CT_{max}) and exhibit a lower optimal temperature (T_{opt}) compared to tadpoles not exposed to the herbicide. Furthermore, because predator cues and the herbicide can induce deeper tails in tadpoles, we hypothesized that tadpoles exposed to either stressor will suffer a vertical shift upward in their TPC across a range of temperatures (Huey and Kingsolver 1989), and have increased swimming performance (Van Buskirk and McCollum 2000).

However, it is also possible that the herbicide will have a negative effect on swimming performance (e.g., Hopkins et al. 2005) if induced morphological changes are countered by other phenotypic changes that impair swimming ability.

Methods

Inducing the tadpoles

The induction experiment was conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology in northwest Pennsylvania, USA. The experiment used a completely randomized, 2 x 2 factorial design comprised of the presence or absence of predator cues crossed with the presence or absence of an herbicide (nominal concentrations of 0 or 2 mg active ingredient per litre (a.e./L). Based on past studies, this herbicide concentration should remain sublethal to gray treefrog tadpoles while inducing morphology changes (e.g., Relyea 2005, Jones et al. 2011).

The four treatment combinations were replicated four times for a total of 16 mesocosms, which consisted of 120-L wading pools, set outdoors (air temperature ranged from 9°C to 28°C), that we filled with 100 L of well water on 11 June 2011. We then added 100 g of dry leaves (*Quercus* spp.) and 5 g of rabbit chow to serve as habitat structure and an initial nutrient source, respectively. We also added an aliquot of zooplankton and phytoplankton that was a mixture from 5 local ponds. Each mesocosm was equipped with a predator cage constructed of 10 x 10 cm well pipe covered with window screen at each end. These cages allow the chemical cues emitted during predation to diffuse through the water while preventing the predators from killing the target tadpoles (Petranka et al. 1987, Kats et al. 1988, Relyea and Auld 2005). Mesocosms were covered with a 60% shade cloth, for the duration of the outdoor experiment.

To obtain tadpoles for the experiment, we collected > 20 amplecting pairs of grey treefrogs from a nearby wetland (41° 34' 9.55" N, 80° 27' 22.29" W) on 18, 21 and 22 May 2011, and allowed them to lay eggs in tubs containing aged well water. Once the eggs hatched, the tadpoles were held in outdoor pools and fed rabbit pellets *ad libitum* until used in the experiment.

On 15 June 2011, which we defined as day 0 of the experiment, we added 40 tadpoles to each mesocosm from a mixture of the clutches with an initial mass (± SE) of 37.5 ± 2.1 mg per tadpole (subsample, N = 20). On day 1, we applied the herbicide treatment. To achieve nominal concentrations of 2 mg a.e./L, we prepared 8 equal mixtures containing 372 µL of stock solution (Roundup Power Max®; concentration = 540 g a.e./L) and 250 ml of water. For the eight mesocosms assigned the herbicide treatment, we drizzled one mixture into each mesocosm. For the eight mesocosms assigned the no-herbicide treatment, we drizzled 250 mL of water into each mesocosm. Approximately 1 hr after dosing, we collected water samples from each tank to confirm the concentration of the herbicide. An independent analysis found that the concentrations in the water were 0 and 1.55 mg a.e./L (Mississippi State Chemical Laboratory, Mississippi State, MS). Observing lower actual concentrations is a common phenomenon in mesocosm experiments (reviewed in Brock et al. 2000), likely as the result of binding to surfaces in the mesocosm and degradation of the samples before the testing is conducted. Jones et al. (2010) measured little herbicide breakdown for a similar time period, so we assumed there was little change in herbicide concentration during the induction experiment.

After sampling the water, we manipulated the predator environment. For mesocosms assigned the no-predator treatment, the cages remained empty. For mesocosms assigned the predator-cue treatment, we placed a single dragonfly nymph (*Anax junius*) inside the predator cage. Each dragonfly was fed ~300 mg of grey treefrog tadpole biomass every 2 d (see Relyea and Auld 2005). Prior to each feeding, we observed no tadpoles left in the predator cage, which indicates that the dragonfly nymphs consumed the tadpoles in the cages. The feeding continued until day 10 to allow tadpole growth and induction by the herbicide and predator cues (see also Annexe III-A).

Determining the critical thermal maximum of the tadpoles

On day 10, we brought sets of tadpoles into the laboratory to allow them to acclimate at a temperature of 20 °C (approximately the average temperature experienced in the mesocosms), with a 12L:12D photoperiod, for 4 to 5 d before testing them for CT_{max} and T_{opt} (Hutchison 1961, Brattstrom 1968; see also Annexe III-B). During acclimation, tadpoles were fed rabbit pellets *ad libitum* and we maintained the predator and herbicide environments to help prevent the loss of any phenotype induction (Relyea 2003). All tested larvae were below Gosner stage 38 (Gosner 1960). This is important because tadpoles close to metamorphic climax exhibit a significant decline in thermal tolerance (Sherman 1980).

Table 3.1. Critical thermal maximum (CT_{max}), sample size (N) and body mass of *Hyla versicolor* tadpoles, in four treatments. Tested tadpoles are representative of the four mesocosms used for each treatment.

Treatment	N	CT_{max} (°C ± SE)	Body mass (mg ± SE)
Control	13	41.78 ± 0.1	483.7 ± 22.9
Predator	13	42.14 ± 0.1	520.4 ± 29.3
Roundup	15	41.76 ± 0.1	545.4 ± 28.0
Predator + Roundup	15	42.17 ± 0.1	489.8 ± 34.2

We obtained upper critical thermal tolerances (CT_{max}) by using a slightly modified version of Hutchison's dynamic method (Lutterschmidt and Hutchison 1997b). We exposed tadpoles to a constant heating rate of 0.05 °C min^{-1} (3ºC h^{-1}), which simulates a natural rate of temperature increase in ponds (H. Duarte, M. Tejedo, J. Hammond, M. Katzenberger, R.A. Relyea, unpublished data from dataloggers; see also Terblanche et al. 2011) until we observed complete immobility, which signaled the endpoint of the experiment. After reaching CT_{max}, we transferred tadpoles to cooler water (~20 °C) to allow recovery. After complete recovery, the tadpoles were weighed and we found that the mass of the tadpoles had increased by 13- to 15-fold since day 0. We tested 3 to 4 tadpoles from each mesocosm, for a total of 56 tadpoles from the 16 mesocosms, as seen in Table 3.1.

We performed an analysis of variance (ANOVA) that used CT_{max} as the dependent variable, predator cues and herbicide as categorical factors (including the interaction of these factors), and mesocosm nested within the interaction of predator cues and herbicide (i.e. mesocosm nested within treatment). Given that tadpole mass was not correlated with CT_{max} (see results), we did not include it as a covariate. No data transformations were required for this analysis.

Determining the thermal performance curves for tadpole burst speed

Locomotor performance, measured as a TPC, is considered to be a proxy of maximum physiological performance and has been used to estimate optimum temperatures in amphibians (Gvoždík and Van Damme 2006, 2008). We obtained TPCs by measuring each tadpole's maximal burst swimming speed (i.e. burst speed) across a range of temperatures. To determine burst speed, tadpoles were placed individually in a portable thermal bath (patent license ES 2372085), which consists of an opened cross section methacrylate tube (1 m long x 6 cm wide x 3 cm deep) filled with water of a given temperature. We then gently prodded the tadpole with a thin stick to stimulate swimming. Each trial was recorded using a digital camera (30 frames/s) positioned above the tube (JVC Everio GZ-MG505). TPCs were defined using a set of six temperatures (20°, 24°, 28°, 32°, 35° and 38°C). This set includes temperatures tadpoles experienced in the mesocosms (20°-32°C) and two more (35° and 38°C) which they might be exposed to in a scenario of increasing environmental temperatures (but lower than their critical thermal maximum). Temperatures were tested in a random order and, for each temperature, tadpoles from the four treatments were tested in the same session; therefore, all treatments had the same temperature order. Prior to swimming, tadpoles were held individually in 250-ml containers at the test temperature for approximately 1 hr. A different set of tadpoles (total N = 570) was used for each temperature (Table 3.2) and each wading pool was represented equally in each set.

Table 3.2. Experimental temperatures, sample size (N), total tadpole length (TTL), and maximum swimming speeds (mean ± SE) for gray treefrog tadpoles that were exposed to predator cues and the herbicide Roundup.

Temp.	Control			Predator			Roundup			Predator + Roundup		
	N	TTL (mm)	Speed (cm/s)	N	TTL (mm)	Speed (cm/s)	N	TTL (mm)	Speed (cm/s)	N	TTL (mm)	Speed (cm/s)
20 °C	22	37.3 ± 0.6	39.2 ± 1.0	24	38.2 ± 0.6	41.1 ± 1.1	23	38.8 ± 0.6	41.0 ± 1.4	24	37.9 ± 0.7	40.2 ± 1.3
24 °C	24	38.1 ± 0.5	41.3 ± 1.1	24	41.0 ± 0.5	46.9 ± 1.5	23	38.8 ± 0.5	44.0 ± 1.3	23	38.0 ± 0.6	44.0 ± 1.3
28 °C	24	39.9 ± 0.6	45.4 ± 1.5	24	42.1 ± 0.5	52.3 ± 1.7	24	41.8 ± 0.5	50.7 ± 1.8	24	39.7 ± 0.6	50.3 ± 1.7
32 °C	24	39.4 ± 0.6	46.7 ± 1.3	24	39.7 ± 0.5	52.5 ± 1.2	24	40.2 ± 0.6	50.2 ± 1.4	24	39.1 ± 0.6	52.5 ± 1.1
35 °C	24	39.6 ± 0.6	45.8 ± 1.7	24	40.8 ± 0.5	51.5 ± 1.6	24	40.6 ± 0.5	47.1 ± 1.7	24	40.4 ± 0.6	50.1 ± 1.8
38 °C	24	37.9 ± 0.5	40.2 ± 1.8	24	37.6 ± 0.6	44.6 ± 1.6	24	39.1 ± 0.6	41.5 ± 2.1	23	36.2 ± 0.6	42.3 ± 2.1

After the tadpole started to move, we used the software Measurement in Motion (Learning in Motion 2004) to estimate burst speed over three frames (0.1 s) by measuring the distance the center of mass moved between frames (Arendt 2009, 2010). After conducting at least three bouts, we used the fastest speed measured for a given tadpole as our measure of that individual's burst speed. Since maximal swimming speed may scale with body size (Gvoždík and Van Damme 2006) and body size may confound the effect of speed on escape success (Van Damme and Van Dooren 1999), we used size-corrected burst speed (using tadpole total length) when constructing TPCs.

To describe the TPCs for burst speed, we used the Template Mode of Variation method (TMV, Izem and Kingsolver 2005; see Annexe III-C) which employs a polynomial function to decompose variation among TPCs into three predetermined modes of variation with biological connotation: vertical shift (faster-slower), horizontal shift (hotter-colder), and specialist-generalist trade-offs (Huey and Kingsolver 1989; see Izem and Kingsolver 2005). Since we tested tadpole performance at six temperatures, we assumed that the common template curve was a fourth-degree polynomial, as in previous studies (e.g., Gvoždík and Van Damme 2008). Making this assumption avoids inadequately describing TPCs, which can happen when using a lower-order polynomial (David et al. 1997, Izem and Kingsolver 2005).

In addition to using the TMV method, we also calculated maximum performance (z_{max}) to evaluate changes in maximum swimming speed at the optimum temperature and a more traditional measurement of performance breadth to confirm specialist-generalist trade-offs (using B_{95}, which is the range of temperatures at which performance values exceed 95% of the maximum; Huey and Stevenson 1979). We used B_{95} instead of the traditional B_{80} because the lower limit of B_{80} would fall below 20°C, which is outside the tested range of temperatures. All computations regarding the TPCs, except for B_{95}, were made using the Matlab code by R. Izem (available online in the appendix of Izem and Kingsolver 2005). We also confirmed the fit of each treatment's curve and calculated standard error (SE) of each curve's parameters using *nlinfit* and *nlparci* functions, respectively, in Matlab (The MathWorks 2013).

We conducted an ANOVA that used burst speed as the dependent variable, temperature, predator cues and the herbicide (including the interaction of these factors) as categorical factors and, mesocosm nested within the interaction of predator cues and herbicide (i.e. mesocosm nested within treatment). ANOVA analysis was followed by a Tukey post-hoc test.

Assessing the morphology of the tadpoles

After the swimming trials, we determined the mass and developmental stage of each tested tadpole. We then took lateral photos of each tadpole and digitized the images for morphometric measurements. We captured the shape of tadpoles by digitizing 10 landmarks and 15 semi-landmarks (see Annexe III-D; see also Dayton et al. 2005, Arendt 2010) on each tadpole using tpsDig2 software (Rohlf 2010a). We then extracted partial warps and the uniform component with tpsRelw software (Rohlf 2010b), which we used as our shape variables in a subsequent analysis. We visualized variation in landmark positions using the thin-plate spline approach (transformation grids, Bookstein 1991) in MorphJ (Klingenberg 2011). As an alternative approach to quantify tadpole morphology, we also took the following linear measurements of each tadpole: total tadpole length (TTL, distance between snout and tip of tail fin), body length (BL, distance between snout and point where bottom edge of tail muscle meets body), body depth (BD, deepest point of the body), tail length (TL, distance between point where bottom edge of tail muscle meets body and tip of tail fin), muscle depth (MD, deepest point of the muscle) and tail depth (TD, maximum depth of the tail fin).

We conducted canonical correlation analysis as a dimension-reducing procedure to obtain two morphological indices (i.e. a linear combination of shape variables); one was for the linear measurements (MI_{lin}) and the other was for the partial warps and uniform component (MI_{geo}). We then examined these two indices for correlations with burst speed (across all treatments; see Dayton et al. 2005). To determine if predator cues, herbicide, and their interaction influenced tadpole size (i.e. centroid) or shape (MI_{lin} or MI_{geo}), we performed three ANOVAs followed by Tukey HSD post-hoc tests; mesocosms were nested within the interaction of predator cues and herbicide (i.e. mesocosm nested within treatment). Shape variables (MI_{lin} and MI_{geo}) and tadpole size (centroid) were then used as continuous predictors, along with temperature, predator cues and herbicide as a categorical predictors, in two ANCOVA analysis (testing either MI_{lin} or MI_{geo} separately), to evaluate their effects on burst speed. We performed all analyses using Matlab (The MathWorks 2013), except when mentioned otherwise, and used a significance level of α = 0.05.

All experiments were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee (Protocol #12050451).

Results

Critical thermal maxima of the tadpoles

In our analysis of CT_{max}, there were no differences among mesocosms within a given treatment. We found an effect of predator cues but no effect of the herbicide or the interaction of both (Table 3.3). Averaged across herbicide treatments, tadpoles exposed to predators had a CT_{max} that was 0.4 °C higher than tadpoles not exposed to predators (Table 3.1). CT_{max} was not correlated with tadpole mass (Pearson's R = -0.17, $p = 0.22$).

Table 3.3. ANOVA using CT_{max} as dependent variable, predator cues and Roundup as categorical factors (including the interaction of these factors) and, mesocosm nested within the interaction of predator cues and Roundup, for *Hyla versicolor*. Univariate tests of significance for CT_{max}. In this model, we used Sigma-restricted parameterization and Type III sum of squares.

	SS	**d.f.**	**MS**	**F**	**p**
Predator	1.993	1	1.993	14.9	< 0.001
Roundup	0.006	1	0.006	0.04	0.834
Predator*Roundup	0.009	1	0.009	0.06	0.801
Mesocosm (Predator*Roundup)	1.329	12	0.111	0.83	0.622
Error	5.350	40	0.134		

Thermal performance curves for tadpole burst speed

When we test tadpole swimming ability across different water temperatures, we found that swimming burst speed varied with temperature (Table 3.2). When we used the TMV method on size-corrected performance data, we obtained both a common template curve, which provided a good approximation of the common shape of each treatment's curve (Figure 3.1), and a three-parameter shape-invariant model (with the use of a fourth-degree polynomial), which explained over 99% of the variation for swimming speed.

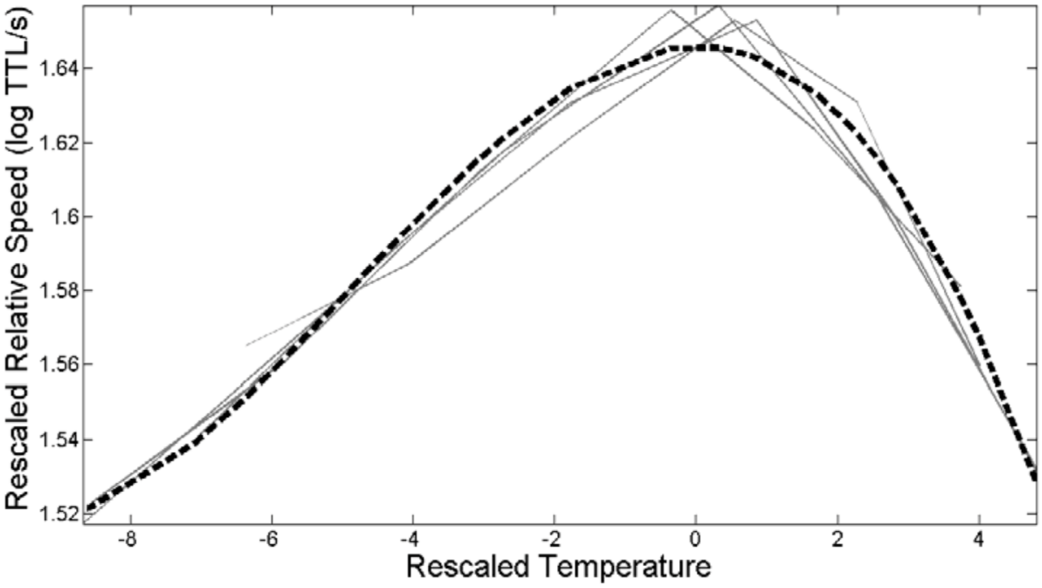


Figure 3.1. Rescaled thermal performance curves for swimming speed in each treatment with fitted common template shape. Common template shape z(T) is represented by a dashed line and the treatments by solid lines. Each thermal performance curve of a treatment (i) and temperature were standardized with respect to the estimates of height (h), location (m; T_{opt}), and width (w) parameters from the fit to model. Rescaled optimum temperature $T_{opt} = 0$. (see [46,51]). Swimming $z(T) = 1.6458 - 0.004T^2 - 0.00023982T^3 + 0.000003493T^4$.

Table 3.4. Parameters of thermal performance curves for maximum swimming speed in four treatments, for *Hyla versicolor*, estimated with TMV method (Izem and Kingsolver, 2005) and nlinfit/nlparci functions in Matlab (Mathworks, 2009)

Treatment	**TMV parameters**						**nlinfit/nlparci**		
	$h^{¥}$	**T_{opt}**	**w**	**z_{max} 1**	**z_{max} 2**	**B_{95}**	**h ± SE**	**T_{opt} ± SE**	**w ± SE**
Control	0.12	31.05	1.74	1.07	11.65	18.36	0.12 ± 0.10	31.07 ± 0.76	1.73 ± 0.19
Predator	-0.01	32.52	1.47	1.11	12.96	14.29	-0.01 ± 0.09	32.53 ± 0.52	1.47 ± 0.13
Roundup	0.00	30.70	1.52	1.09	12.27	14.97	0.00 ± 0.09	30.71 ± 0.65	1.52 ± 0.14
Predator + Roundup	-0.11	31.58	1.34	1.12	13.13	12.35	-0.11 ± 0.09	31.58 ± 0.51	1.33 ± 0.11

$h^{¥}$, height (log TTL/s); T_{opt}, optimal temperature (°C); w, width (dimensionless); z_{max} 1 (TPC), maximum performance (log TTL/s); z_{max} 2 (TTL/s), maximum performance (TTL/s); B_{95}, thermal performance breadth (°C).

Decomposition of the total variation into the three pre-determined directions of variation reveals that TPCs for swimming speed vary mostly in the specialist-generalist (53.27%) direction and the vertical (45.98%) direction, but very little in the horizontal (0.59%) direction. This indicates that tadpoles in the control treatment had a wider swimming TPC than tadpoles exposed to predator cues or the herbicide, even when comparing more traditional measures of curve width (B_{95}; Table 3.4, Figure 3.2). Thus, most of the variation in the TPCs is due to specialist-generalist trade-offs and differences in overall performance (faster-slower), rather than changes in T_{opt} (hotter-colder). Indeed, tadpoles raised in the herbicide treatment exhibited only a small decrease in T_{opt} (-0.4 °C) while tadpoles raised with predator cues exhibited an increase in T_{opt} (1.5 °C). Tadpoles raised with both predators and herbicide exhibited a T_{opt} that was intermediate in magnitude between the latter two treatments but still higher (0.5 °C) than tadpoles raised in the control treatment. The only significant difference in T_{opt} was between tadpoles exposed only to herbicide and those exposed only to predator cues (1.8 °C; 2-tailed *t*-test, $p < 0.05$). Maximal performance (z_{max}) was marginally correlated with performance breadth (Pearson's R = -0.95, $p = 0.051$).

Temperature and predator cues both influenced burst speed (Table 3.5). There was also a significant interaction between predator cues and herbicide. Tadpoles in the control treatment had slower burst speeds across all temperatures than tadpoles in the other three treatments (all $p < 0.05$). Tadpoles raised in the predator treatment were also faster than those from herbicide treatment ($p < 0.05$). Furthermore, tadpoles in all treatments containing predator cues or herbicide had higher maximum performance (z_{max}) than tadpoles in the control treatment, so that their burst speed at the optimum temperature was higher than the burst speed of tadpoles raised without any cues. These differences in the parameters of the TPCs can be seen as changes in the overall shape of the curves (Figure. 3.2). Our analysis of burst speed also revealed a significant effect of mesocosms (nested within treatment), however the magnitude of this effect was much smaller than in other effects, such as the interaction of predator cues and herbicide (Table 3.5). Nevertheless, we checked for burst speed differences among tanks of the same treatment and temperature and we found no significant effect of mesocosm on burst speed, in any of the treatment-temperature combinations (all $p > 0.05$).

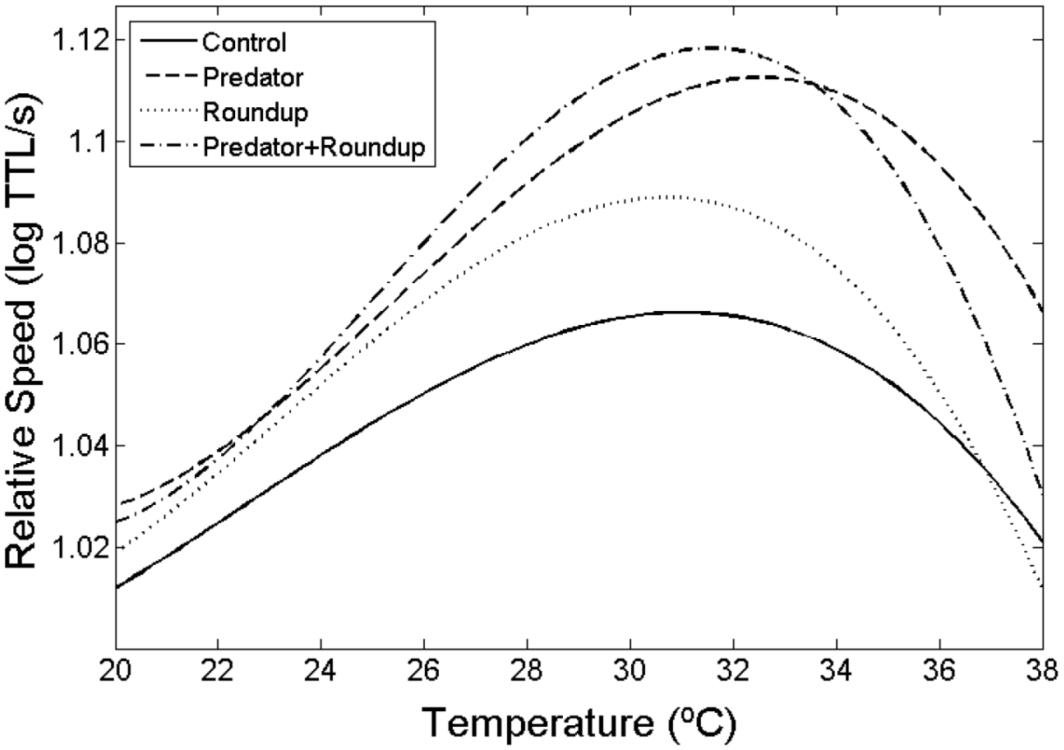


Figure 3.2. Overall shape of the thermal performance curves for each of the four induction treatments. Each treatment is represented by a thermal performance curve for tadpole swimming speed: control - solid line, predator - dashed line, Roundup - dotted line and predator+Roundup - dash-dot line.

Induced morphology of the tadpoles

We observed size and shape changes in tadpoles exposed to the herbicide and predator cue treatments (Figure. 3.3). Predator cues and herbicide had no main effects on tadpole centroid size (Table 3.6a) but they did have a significant interaction; tadpoles exposed to predator cues + herbicide were smaller than those exposed only to the herbicide or only to the predator cues (both $p < 0.05$). Similarly, tadpoles in the control treatment were smaller than those exposed only to the herbicide or only to the predator cues (both $p < 0.05$). For geometric morphometric measurements, both predator cues and herbicide influenced tadpole shape (Table 3.6b) and there was a significant interaction between the two factors. Tadpoles raised in the control treatment differed from those raised in the other three treatments (all $p < 0.05$), however these did not differ amongst themselves. For linear measurements, only predator cues significantly influenced shape of tadpoles (Table 3.6c). Tadpoles raised in predator or predator + herbicide treatment differed from those raised in herbicide or control treatments (all $p < 0.05$). Mesocosm effect on either centroid or shape (MI_{lin} or MI_{geo}) was non-significant (Table 3.6).

Table 3.5. ANOVA using burst speed as dependent variable, and temperature, mesocosm, predator cues and Roundup as categorical predictors, with mesocosm nested within the interaction of predator cues and Roundup, for *Hyla versicolor*. Univariate tests of significance for burst speed. We used Sigma-restricted parameterization and Type III sum of squares.

	SS	d.f.	MS	F	p
Temperature	0.891	5	0.178	32.17	<0.001
Predator	0.106	1	0.106	19.16	<0.001
Roundup	0.002	1	0.002	0.38	0.537
Predator*Roundup	0.070	1	0.070	12.65	<0.001
Mesocosm (Predator*Roundup)	0.127	12	0.010	1.92	0.03
Predator*Temperature	0.023	5	0.005	0.83	0.528
Roundup*Temperature	0.017	5	0.003	0.62	0.683
Predator*Roundup*Temperature	0.009	5	0.002	0.33	0.903
Error	3.085	546	0.006		

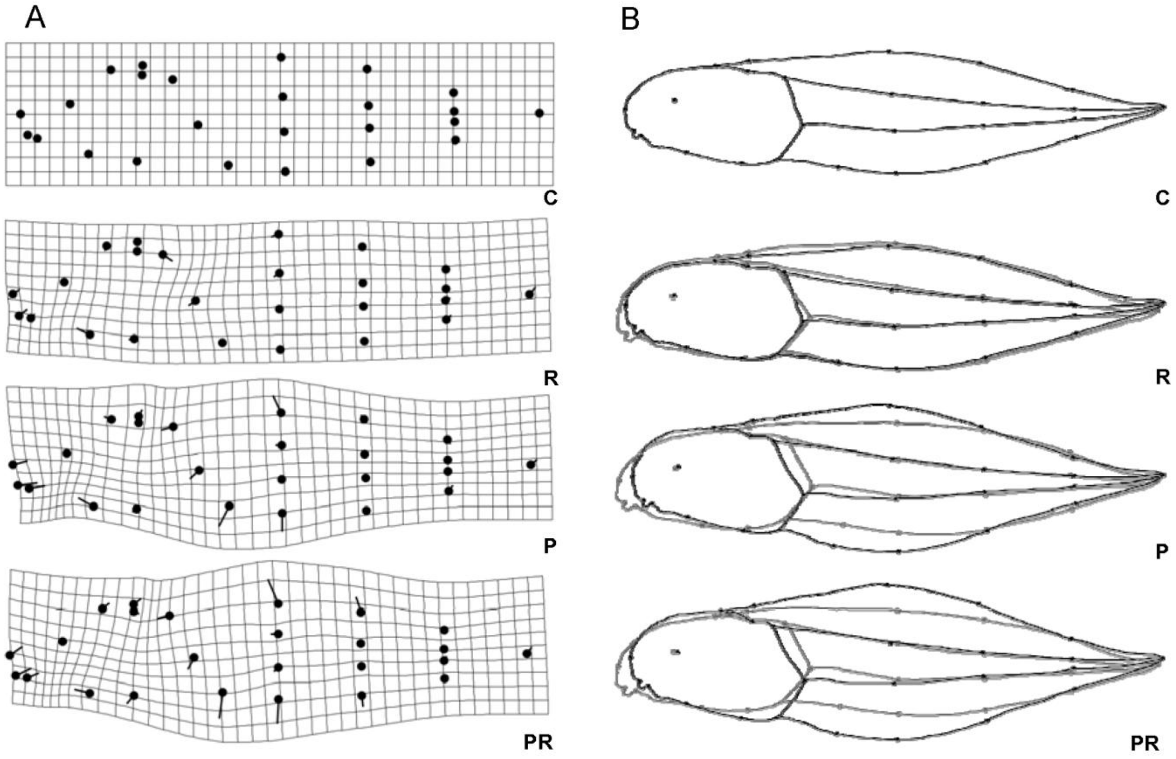


Figure 3. Transformation grids with landmarks and warped outline drawings for each treatment's tadpole shape. a) Transformation grids with landmarks (black dots) and vectors showing direction of variation; b) comparison of warped outline drawings for each treatment shape (black) and control shape (grey). Transformation grids and warped outline drawings were magnified (x5) to better illustrate the differences. C – Control, R – Roundup, P – Predator and PR – Predator + Roundup.

Table 3.6. ANOVAs to determine if predator cues and Roundup (including their interaction) influenced size (a; centroid), or shape (b and c) of tadpoles (MI_{geo}, for geometric morphometric measurements, or $MI_{lin,}$ for linear measurements, respectively) with mesocosm nested within the interaction of predator cues and Roundup (i.e. mesocosm nested within treatment). We used Sigma-restricted parameterization and Type III (Effective hypothesis) sum of squares.

a) Centroid (size)	**SS**	**d.f.**	**MS**	**F**	**p**
Predator	19.9	1	19.91	0.97	0.326
Roundup	4.3	1	4.32	0.21	0.647
Predator*Roundup	521.7	1	521.7	25.38	< 0.001
Mesocosm (Predator*Roundup)	423.0	12	35.25	1.72	0.06
Error	11386.2	554	20.55		

b) MI_{geo} (shape)	**SS**	**d.f.**	**MS**	**F**	**p**
Predator	11.77	1	11.766	12.21	< 0.001
Roundup	5.17	1	5.172	5.37	0.021
Predator*Roundup	5.68	1	5.684	5.90	0.016
Mesocosm (Predator*Roundup)	12.37	12	1.031	1.07	0.383
Error	533.95	554	0.964		

c) MI_{lin} (shape)	**SS**	**d.f.**	**MS**	**F**	**p**
Predator	29.35	1	29.348	30.95	< 0.001
Roundup	3.32	1	3.317	3.50	0.062
Predator*Roundup	2.46	1	2.463	2.60	0.108
Mesocosm (Predator*Roundup)	8.67	12	0.723	0.76	0.690
Error	525.32	554	0.948		

Table 3.7. ANCOVA analysis using burst speed as dependent variable, shape variables MI_{geo} (a) or MI_{lin} (b) and tadpole size (centroid) as continuous predictors, alongside temperature, predator cues and Roundup as categorical predictors. Univariate tests of significance for burst speed. In both models, we used Sigma-restricted parameterization and Type III (Effective hypothesis) sum of squares.

a)	**SS**	**d.f.**	**MS**	**F**	**p**
Predator	0.068	1	0.068	15.01	< 0.001
Roundup	0.000	1	0.000	0.01	0.909
Temperature	0.197	5	0.039	8.66	< 0.001
Size (Centroid)	0.167	1	0.167	36.70	< 0.001
Shape (MI_{geo})	0.129	1	0.129	28.27	< 0.001
Predator*Roundup	0.011	1	0.011	2.34	0.127
Error	2.544	559	0.005		

b)	**SS**	**d.f.**	**MS**	**F**	**p**
Predator	0.101	1	0.101	21.24	< 0.001
Roundup	0.003	1	0.003	0.59	0.443
Temperature	0.507	5	0.101	21.30	< 0.001
Size (Centroid)	0.410	1	0.410	86.09	< 0.001
Shape (MI_{lin})	0.011	1	0.011	2.37	0.124
Predator*Roundup	0.012	1	0.012	2.45	0.118
Error	2.661	559	0.005		

Overall, compared to tadpoles in the control, tadpoles in the other three treatments exhibited relatively shorter bodies. Furthermore, in the two treatments containing predator cues, tadpoles exhibited an increase in their relative tail length and tail depth (Figure. 3.3). Apart from temperature and predator cues, burst speed was also influenced by tadpole's size, either when using morphometric geometric data (Table 3.7a) or linear measurements (Table 3.7b). We also found a significant effect of shape on burst speed when using geometric morphometric data (Table 3.7a).

Discussion

We discovered that predator cues and the herbicide Roundup can affect the thermal physiology of *Hyla versicolor* tadpoles. Predator cues induced tadpoles to have CT_{max} values that were 0.4 °C higher whereas the herbicide had no effect. Predator cues and Roundup also influenced the shape of the thermal performance curves, resulting in changes in optimum temperature, performance breadth and maximal performance (Figure 3.2). Furthermore, predator cues also induced morphological changes that increased the tadpoles' burst speed.

Roundup, a glyphosate based broad-spectrum systemic herbicide, did not have any effect on CT_{max} estimates of tadpoles. However there have been reports of other contaminants affecting the thermal physiology of vertebrates. Among insecticides, for example, endosulfan (an organochlorine insecticide that affects the central nervous system) and chlorpyrifos (an organophosphate insecticide that inhibits acetylcholinesterase) are known to decrease CT_{max} in fishes (Patra et al. 2007). Other environmental contaminants, such as cadmium and copper, can adversely affect the ability of fish to withstand high temperature stress (Carrier and Beitinger 1988, Lydy and Wissing 1988). Whether all of these observations in fishes can be extrapolated to other species of aquatic organisms, such as tadpoles, is yet to be determined. Based on these studies and our own results, it seems that the effects of pesticides on CT_{max} may depend on the type of pesticide, the concentration of the pesticide, and how it affects the organism (i.e. its mode of action). There is the possibility that using higher concentrations of the herbicide might induce a decrease in CT_{max}, but higher concentrations will cause tadpole death

(Relyea and Jones 2009). Furthermore, the herbicide also did not interfere with the increase in CT_{max} induced by predator cues; tadpoles exposed to predator cues + herbicide had similar CT_{max} values to those exposed only to predator cues.

Different methodological protocols and biological sources can affect estimates of upper thermal tolerances (see Navas et al. 2008, Terblanche et al. 2011). For example, the ramping rate used (Terblanche et al. 2007, Chown et al. 2009a, Mitchell and Hoffmann 2010, Rezende et al. 2011), the selection of end-point (Lutterschmidt and Hutchison 1997b), variations in previous thermal acclimation (Brattstrom 1968), ontogenetic stage (Sherman 1980), time of day, and photoperiod (Mahoney and Hutchison 1969) all may promote shifts in amphibian upper thermal tolerances. We discovered that predatory cues can also affect CT_{max} estimates of prey. An increase in thermal tolerance of predator-induced tadpoles would cause an increase in their warming tolerance, which is the difference between CT_{max} and maximum temperature of the environment to which an ectotherm is exposed (Deutsch et al. 2008, Duarte et al. 2012). This means that tadpoles exposed to predator cues would be less susceptible to acute thermal stress than tadpoles that were not exposed to predator cues. In contrast, an exposure to the herbicide, at least at the concentration used in our study, would not affect the warming tolerance of tadpoles.

An exposure to predator cues and the herbicide had interactive effects on tadpole burst speed. The interaction occurred because the herbicide alone and predator cues alone each increased burst speed compared to the control, but the combination of the herbicide and predator cues induced an increase that was not larger than predator cues alone. Therefore, since the combination of the herbicide and predators cues was not additive, in the presence of predator cues, exposure to the herbicide caused no change in burst speed.

The presence of either predator cues or the herbicide narrowed the performance breadth of the TPC while increasing maximal performance. As performance breadth is negatively correlated with maximal performance, we would expect a generalist-specialist trade-off. Tadpoles from a treatment which induced a more specialist curve (as demonstrated by predator cues + herbicide) would perform better at the optimum temperature but gradually decrease in performance, as moving away from the optimum temperature, until reaching a point where tadpoles from a treatment which induced a more generalist curve (as demonstrated by control) would outperform them (see Huey and Kingsolver 1989, Izem and Kingsolver 2005; Table 3.4). However, we do not see a decline in performance at the extremes of the thermal performance curve, at the tested temperatures, as a result of this trade-off. This observation is confirmed by thermal tolerance data where none of the tadpoles raised in any of the treatments with predator cues or the herbicide had lower CT_{max} than those from the control treatment. Instead, it appears the expected decline in sub-optimal performance resulting from a generalist-specialist trade-off is compensated by the increase in overall performance, so that tadpoles raised in the control treatment always perform, on average, worse than herbicide- or predator-induced tadpoles, at least at the tested temperatures. Therefore, when comparing thermal performance curves, the resulting increase in overall performance was asymmetric, being greater around the optimum temperature and lower at the extreme temperatures.

Surprisingly, predator cues and the herbicide also produced changes in the optimum temperature, but in opposite directions. Of course, the small decrease in optimum temperature caused by the herbicide (0.4 °C) may have little or no biological relevance. In contrast, the increase in optimum temperature promoted by predator cues (approximately 1.5 °C) may be important, especially when new assessments suggested that environmental impacts will require smaller degrees of global warming than previously thought (Smith et al. 2009). Since predator cues increase optimum temperature, the difference between optimum temperature and the environmental temperature should also increase (i.e. thermal safety margins (TSM); see Deutsch et al. 2008), which would be beneficial to the tadpoles in the current scenario of increasing global temperatures.

Previous studies have demonstrated that changes in the shape or position of thermal performance curves can occur due to acclimation (e.g., Kingsolver and Huey 1998, Lachenicht et al. 2010, Condon et al. 2010) or that thermal performance curves of different locomotor strategies for the same organism can have different shapes (e.g., Gvoždík et al. 2007, Gvoždík and Van Damme 2008). In the present study, we demonstrate that the presence of sublethal concentrations of an herbicide and cues from predators can also produce changes in the thermal performance curves and therefore affect how tadpoles respond to environmental temperature changes.

Although it has been documented that predators can affect the behavioural thermoregulation of their prey (e.g., Angilletta 2009), to our knowledge this is the first study to demonstrate a predator altering the thermal physiology of their prey by increasing CT_{max}, increasing the optimum temperature, and producing changes in the thermal performance curves. It has also been demonstrated that Roundup's lethality increases with competition stress (Jones et al. 2011) and that predator cues can improve tadpole survival when tadpoles are exposed to the herbicide under stratified water conditions (Relyea 2012). Therefore, one could make the argument that acclimation to predator cues might be beneficial under warmer temperatures. However, we should also keep in mind that predation simultaneously has a negative effect on tadpole populations and can select for particular phenotypes (see Relyea 2002). To display a predator-induced phenotype, tadpoles need to detect chemical cues that are released when other tadpoles (particularly conspecifics) are consumed. So, the possible positive effects of predator cues on the thermal physiology, in a global warming scenario, would only be beneficial for those phenotypes that survive predation.

Predator cues in our study induced morphology changes (relative smaller bodies, deeper tails and deeper tail muscle) that were similar to those observed in previous studies (e.g., Relyea 2001). These morphological changes likely explain why tadpoles exposed to predator cues swam faster than control tadpoles. Exposure to the herbicide (see Figure 3.3) induced relative smaller bodies, and the observed changes partially resembled the predator-induced phenotype (see also Relyea 2012). The induction of relatively deeper tadpole tails by the herbicide was less evident in the current work than in the study of Relyea (2012). However, this may be due to a number of differences in the experimental protocol including the duration of exposure and a substantially different experimental venue.

Predator cues and the herbicide caused interactive effects on tadpole size. Tadpoles exposed to predator cues + herbicide were smaller than those exposed only to the herbicide or only to predator cues. Tadpoles raised in the control treatment also tended to be smaller than those exposed only to the herbicide or only to predator cues. This may explain why tadpoles from the herbicide treatment also swam faster than tadpoles from the control treatment. As a result, all three treatments had better overall swimming performance than in control, with increase in burst speed related to the magnitude of morphology change (more induction, higher performance) and size. Furthermore, predator-induced morphology changes can be reversed if cues are removed (Relyea 2003). As a result, some of the changes in the thermal performance curve may also be reversible. If so, in the absence of cues, the predator- and herbicide-induced TPC shapes would revert back to the original curve (i.e. the control curve).

The mechanism underlying the ability of the herbicide to induce morphological changes in tadpoles is still unknown. It has been suggested that the herbicide may be interfering with the stress hormones that induce anti-predator defences (Glennemeier and Denver 2002) or that herbicides and predator cues activate shared endocrinological pathways (Relyea 2012). We have demonstrated that predator cues and the herbicide can affect the thermal physiology of tadpoles, although not all changes occur in the same direction. However, the mechanisms behind these thermal physiology changes are also unknown, with possible scenarios arising from our results: a) herbicide interferes only with the stress hormones that induce anti-predator defenses; b) they do not share the same physiological pathways, or at least not all of them; c) they both activate shared endocrinological pathways but predator cues also indirectly activate temperature-stress response mechanisms; or d) stress response mechanisms are more general than previous thought and predator-induced stress produces similar physiological responses as temperature-induced stress.

Conclusions

Apart from inducing morphology changes, predator cues promoted an increase in CT_{max} and optimum temperature of *Hyla versicolor* tadpoles. As such, in the presence of predators, we can expect tadpoles to have greater warming tolerance and broader thermal safety margins. These changes might indirectly help tadpoles cope with increasing environmental temperatures. The herbicide Roundup is not only toxic to amphibians (and lethal over certain concentrations), but it also produces changes in morphology (Relyea 2012). With this work, we now know that it also interferes, to some extent, with the thermal physiology of tadpoles (in particular in the thermal performance curves), although the effect on warming tolerance and thermal safety margins appears to be marginal. However, Roundup is just one of hundreds of chemicals currently used in anthropogenic activities (e.g., agriculture) and tadpoles can face predation by a wide variety of predator species. Because combinations of pesticides, which are a common situation in natural environments, can have greater impacts than each pesticide alone (Relyea 2009), future studies should test whether combinations of pesticides and predators could have different effects on the thermal physiology of organisms.

In the current scenario of climate change, it is important that we understand the physiological mechanisms underlying tolerance to abiotic stress (Gilman et al. 2006, Tewksbury et al. 2008) and the sensitivity of organisms to changes in the environment (Porter and Gates 1969, Gilman et al. 2006). However, it also is important that we understand the indirect effects of physiological responses (in particular thermal physiology) on species interactions, such as predation, competition and disease transmission (Helmuth et al. 2010). Therefore, understanding the plasticity of thermal performance curves and thermal limits (CT_{max} and CT_{min}) and how these parameters are altered by environmental stressors may be critical to understanding how physiological variation can influence a species' response to climate change (Buckley 2008).

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CHAPTER 4

Vulnerability to climate change across life-stages in amphibian species

Resumen

En el escenario actual de cambio climático, los impactos ecológicos dependerán tanto de la magnitud y el patrón del cambio como de la sensibilidad térmica de los propios organismos. Esa información se hace crucial para entender mejor los mecanismos relacionados con la función fisiológica y el desempeño ecológico. Como la metamorfosis puede romper las relaciones genéticas y de desarrollo entre los rasgos expresados en diferentes etapas del ciclo de vida de los anfibios, en este trabajo estudiamos las curvas de desempeño térmico (incluyendo la temperatura óptima) para determinar si la adaptación al ambiente térmico en la fase larval (renacuajo) puede influir en la fisiología térmica de la especie en la fase juvenil y si hay una diferencia en su vulnerabilidad al cambio climático. Nuestros resultados indicaron que en el desempeño de saltos de los juveniles, algunos rasgos (temperatura óptima y máximo desempeño) parecen mostrar latencia o no están completamente desvinculados de la etapa de vida anterior, mientras que otros (amplitud de desempeño) no mostraron relación entre las fases larval y juvenil. Además, también observamos compensaciones entre las características del desempeño térmico. Los juveniles parecen ser más vulnerables al aumento de las temperaturas ambientales, una vez que la mayoría de las especies ya experimentan temperaturas ambientales por encima de su temperatura óptima en esta etapa de vida. Sin embargo, estos resultados deben ser interpretados con cuidado ya que otros factores pueden afectar directamente el desempeño o interactuar con la temperatura y causar cambios en las curvas de desempeño térmico. Además, las futuras predicciones de la vulnerabilidad de los anfibios al cambio climático también deben incorporar información sobre la termorregulación medioambiental, así como estimaciones de las consecuencias energéticas de la permanencia prolongada en refugios térmicos.

Palabras clave: temperatura óptima, cambio climático, curvas de desempeño térmico, márgenes de seguridad térmica, desacoplamiento adaptativo, ciclos de vida complejos.

Abstract

In the current climate change scenario, ecological impacts will depend on both the magnitude and pattern of climate change and the thermal sensitivity of the organisms in question. To provide a fair vulnerability assessment, it is crucial to understand the mechanisms relating physiological function and ecological performance. In organisms with complex life-cycles, such as amphibians, metamorphosis may break the developmental and genetic relationships between traits expressed at different life stages. Here, we studied thermal performance curves (including optimum temperature) to determine if adaptation to the thermal environment in the tadpole stage can influence the species' thermal physiology in the juvenile stage and if there is a difference in their vulnerability to climate change. Our results indicate that in juvenile jumping performance, some traits (optimum temperature and maximum performance) appear to show latency or not be completely decoupled from the previous life-stage, while others (performance breadth) showed no relation between tadpole and juvenile stages. In addition, we also observed trade-offs between traits of the thermal performance curve. Juveniles appear to be more vulnerable to increasing environmental temperatures, as most species already experience environmental temperatures above their optimum temperature in this life-stage. However, these results should be carefully interpreted as other factors can directly affect performance or interact with temperature and promote changes in the thermal performance curves. In addition, future predictions of amphibian vulnerability to climate change should also incorporate information on behavioral thermoregulation (heat stress avoidance) as well as estimates of the energetic consequences of extended thermal retreats.

Keywords: optimum temperature, climate change, thermal performance curves, thermal safety margins, adaptive decoupling, complex life-cycles

Introduction

In the current scenario of climate change, predictions by the IPCC for the coming decades include a five-fold increase in warming rate, an increase of extreme climatic events (Schär et al. 2004, Diffenbaugh and Ashfaq 2010) and, alteration of global precipitation patterns (IPCC 2007). As ecological impacts will depend on both the magnitude and pattern of climate change and the thermal sensitivity of the organisms in question (Helmuth et al. 2005, Tewksbury et al. 2008, Kingsolver 2009; but see Williams et al. 2008), it is crucial to better understand the mechanisms relating physiological function and ecological performance and to identify the species currently exposed to higher risk of suffering physiological stress (Somero 2005, Helmuth et al. 2010). Hence, forecasting biological responses to current climatic changes emphasizes the necessity of understanding how a species' physiological characteristics vary through space and time (Kearney and Porter 2009, Helmuth et al. 2010), and assess their potential to face these changes via either plasticity or evolution (Pörtner and Farrell 2008, Chown et al. 2010).

Ectotherms constitute the majority of current biodiversity and their basic physiological functions (e.g. locomotion, growth, or reproduction) are strongly influenced by environmental temperature, which makes them especially vulnerable to climate warming. In ectotherms, most physiological processes operate within the bounds of lethal temperature extremes and proceed rapidly over a range of body temperatures defining a thermal performance curve or TPC (see figure 1 in Huey and Stevenson 1979). The performance of a physiological trait gradually increases with temperature from a minimum critical temperature (CT_{min}) to an optimum before dropping precipitously as temperature approaches a critical thermal maximum (CT_{max}). Temperatures either below or above the range of tolerance, defined by the critical thermal limits, result in impaired physiological function (Hillman et al. 2009). As animals often perform under sub-optimal environmental conditions (Huey et al. 1989), there has been increasing interest not only on maximal performance capacity but also on the shape and position of the reaction norm describing the environmental dependence of physiological performance (Schlichting and Pigliucci 1998, Angilletta et al. 2003, Angilletta 2006, Kingsolver et al. 2007, Gvoždík and Van Damme 2008). In the current climate change scenario, the study of thermal sensitivity of ectotherms has received renewed attention, in particular amphibians (Deutsch et al. 2008, Duarte et al. 2012, Sunday et al. 2014).

Amphibians are considered the most globally threatened group of vertebrates (Wake and Vredenburg 2008, Hoffmann et al. 2010) and have a number of physiological, ecological and life-history characteristics that make them highly susceptible to environmental change such as ectothermy, permeable skin and complex life- cycles (Wells 2007). For organisms that experience different selective environments during their development (such as amphibians do), genetic correlations between ontogenetic stages can mean that selection on a trait at one stage may induce a maladaptive change in the same trait at other stages (Watkins 2001). A change in selective environment during the life-cycle can be accompanied by metamorphosis (Wilbur 1980). This process occurs in the amphibian's life-cycle, and it is presumed to be an adaptation to the sequential occupation of temporary wetlands and terrestrial environments (Wells 2007). Metamorphosis is commonly seen as being beneficial since it may break the developmental and genetic relationships between traits expressed at different stages (Ebenman 1992, Moran 1994), and thereby allow the pre- and postmetamorphic stages to adapt independently to their respective environments (Watkins 2001); a view that has been called the adaptive decoupling hypothesis (Moran 1994, Watkins 2001).

In this work, we study two stages of the amphibian life-cycle (aquatic larvae and terrestrial juveniles) to determine if adaptation to the thermal environment in one stage can influence the species' thermal physiology in another stage. We address this question by comparing the thermal physiology of the tadpole and juvenile stages, using thermal performance curves to estimate optimum temperature and other related physiology traits. Thermal physiology traits such as CT_{max} and optimum temperature have been shown to be evolutionarily correlated with environmental temperature (Duarte et al. 2012, Chapter 1), reflecting species adaptation to their thermal habitat. As tadpoles and juveniles occupy two different habitats, and metamorphosis occurs between these two stages, we expect adaptation to the thermal environment to be stage-independent and the thermal performance curve to be adjusted for each life-stage.

By describing the relation between temperature and organism fitness (Huey and Stevenson 1979, Frazier et al. 2006), thermal performance curves also provide a physiological framework which can be used to elucidate fundamental components of the impact of global climate change, albeit in a spatially explicit and empirically constrained way (Deutsch et al. 2008). Hence, we compare thermal safety margins (see Chapters 1 and 2) of tadpoles and juveniles to determine if there is a life-stage that may be more vulnerable to suffer long-term chronic effects from increasing environmental temperatures, such as diminished physiological, developmental or behavioral performance at higher temperatures.

Methods

We sampled larvae of Iberian amphibian species from different locations (Annexe IV-A) and transported them to the laboratory at EBD-CSIC, Seville (Spain). In addition, adults of *S. couchii* and *S. multiplicata* were collected from Arizona and New Mexico states (USA) and shipped to the laboratory in Seville, where they were hormonally stimulated to breed (for details, see Buchholz and Hayes 2002, Kulkarni et al. 2011).

Prior to the experiments, all larvae were maintained at constant room temperature of 20 °C and photoperiod of 12L:12D. Tadpoles were fed rabbit chow *ad libitum* only until metamorphosis was reached, as during tail absorption tadpoles usually do not feed. From the start of tail rebsorption to the end of the performance trials, juveniles were kept individually in plastic containers, maintaining a humid environment and the same light and temperature conditions as before.

To assess the environmental temperatures to which these species are exposed to during their terrestrial stage, we mapped a set of global climate layers (climate grids) available in WorldClim (Hijmans et al. 2005), using QGIS software. We then extracted estimates of T_{avg} (average temperature), T_{max} (maximum temperature), T_{min} (minimum temperature) and, DTF_{annual} (annual diel temperature fluctuation; Annexe IV-A) for each the collection site. Since most of the species studied take at least one year to reach adult size/maturity, we extracted thermal information considering all months of the year. We used Pearson's linear correlation to assess the relation between environmental variables and the thermal physiology of juveniles.

Thermal physiology of juveniles

We estimated thermal sensitivity of juveniles using thermal performance curves based on locomotor performance (maximum jumping distance). Locomotor performance has been employed as a good proxy to estimate optimal temperatures in ectotherms since it may correlate with fitness (Jayne and Bennett 1990, Le Galliard et al. 2004, Husak 2006). To determine maximum jumping distance, juveniles were placed individually on a arena inside a climatic chamber set at 70% humidity and the desired test temperature. We then gently prodded the specimen with a thin stick to stimulate jumping. Each trial was recorded using a digital camera (30 frames/s) positioned above the arena (JVC Everio GZ-MG505). TPCs were defined using a set of temperatures that were tested in a random order. As species differ in their range of performance, to obtain a complete performance curve, temperature sets were adjusted by adding colder or hotter test temperatures when required, as seen in Annexe IV-B. Prior to jumping, juveniles were submitted for approximately half an hour to the test temperature and each individual had two performance trials at each temperature, with a 15 minute interval between them.

Each trial had several jumps and we considered the best jump of both trials as our final measure of maximum jumping distance. Since locomotor performance may scale with body size (Gvoždík and Van Damme 2006) and body size may confound it effect on escape success (Van Damme and Van Dooren 1999), we used size-corrected maximum jumping distance (SVL) for constructing thermal performance curves (Annexe IV-B). To describe the TPCs for maximum jumping distance, we used the Template Mode of Variation method (TMV; Izem and Kingsolver 2005) . Since we tested juvenile performance at several temperatures, we assumed that the common template curve was a fourth-degree polynomial, as in previous studies (Gvoždík and Van Damme 2008, Katzenberger et al. 2014). For each species, we also calculated maximum performance (z_{max}) and performance breadth B_{80}, (range of temperatures at which performance values exceed 80% of the maximum; Huey and Stevenson 1979).

We assessed juveniles' vulnerability to chronic thermal changes using the thermal safety margins (TSM) metric from Deutsch et al. (2008), defined as the difference between the species’ thermal optimum (T_{opt}) and its current (mean) environmental temperature (T_{avg}). We also estimated a second measurement of thermal safety margins (TSM_{max}) which we used maximum (T_{max}) instead of average environmental temperature. We then compared juveniles' thermal physiology and their thermal safety margins to those determined for the tadpole life stage, using paired t-tests (or Wilcoxon signed rank tests when necessary). Note that two estimates of TSM for the tadpole stage were previously calculated, one using environmental information from dataloggers and the other from WorldClim (see Chapters 1 and 2). We used Pearson product-moment correlations (or Spearman's rank correlation, when necessary) to determine if species' physiology traits were maintained in the transition from tadpole to juvenile life stages. As data was collected across multiple species, we also conducted these analysis incorporating phylogenetic information (Felsenstein 1985, Garland Jr. et al. 1992), using phylogenetic generalized least squares (PGLS) analysis under a Brownian motion model of evolution, in package caper (Orme et al. 2013) for R (R Development core team 2014). Hence, we extracted a phylogenetic tree for the sampled species from Pyron and Wiens (2011), keeping both topology and branch lengths estimated in that work. We used also used Pearson product-moment correlations and PGLS analysis to evaluate the relation between physiological measurements and environmental temperature data. We also used *phylosig* in package *phytools* (Revell 2012) for R to determine if thermal physiology traits exhibit phylogenetic signal (Pagel's λ). All analysis reported were performed in Matlab (The MathWorks 2013), except when mentioned otherwise, and were conducted on a significance level of α=0.05.

Results

Using the TMV method, we estimated thermal performance curves for 10 species (Annexe IV-C) and determined the physiology traits associated with them (T_{opt}, z_{max} and B_{80}; Table 4.1). Species with higher optimum temperature showed lower maximum performance (z_{max}; Pearson’s r = -0.71, p = 0.014; PGLS, λ = 0.0, β = -0.33 ± 0.11, t = -3.03, p = 0.014) and higher upper (B_{80max}; Pearson’s r = 0.95, p < 0.00; PGLS, λ = 0.19, β = 0.88 ± 0.09, t = 9.67, p < 0.001) and lower (B_{80min}; PGLS, λ = 0.99, β = 0.65 ± 0.11, t = 5.98, p < 0.001) limits of performance breadth. Furthermore, species with higher maximum performance have narrower performance breadths (B_{80}; Pearson’s r=-0.63, p=0.039; PGLS, λ = 0.0, β = -0.99 ± 0.41, t = -2.42, p = 0.038) and lower B_{80} upper limit (B_{80max}; Pearson’s r=-0.78, p=0.004; PGLS, λ = 0.14, β = -1.48 ± 0.41, t = -3.62, p = 0.006). Performance breadth is also related to both its upper (B_{80max}; Pearson's r=0.63, p=0.037; PGLS, λ = 0.14, β = 0.73 ± 0.32, t = 2.27, p = 0.049) and lower limits (B_{80min}; Pearson's r=-0.62, p=0.042; PGLS, λ = 0.93, β = -0.54 ± 0.19, t = -2.81, p = 0.020).

Table 4.1. Thermal physiology traits for jumping performance and estimates of Thermal Safety Margins for juveniles of the studied amphibian species.

Species	T_{opt}¥	z_{max}	B_{80}	B_{80max}	B_{80min}	TSM	TSM_{tmax}
Alytes dickhilleni	29.57	16.22	12.41	34.00	21.59	18.27	0.37
Alytes muletensis	33.23	15.51	10.38	37.11	26.73	17.63	5.53
Discoglossus jeanneae	29.29	22.79	9.19	32.77	23.58	13.29	-4.51
Hyla arborea	33.09	12.89	12.81	37.73	24.93	16.49	5.39
Hyla meridionalis	31.31	17.42	12.65	35.86	23.22	15.61	-3.69
Pelobates cultripes	31.95	14.61	15.74	37.30	21.56	16.05	-1.85
Pelodytes ibericus Cabra	29.66	12.22	14.09	34.47	20.37	15.56	-2.94
Pelodytes ibericus Toba	29.71	12.69	15.31	35.49	20.18	14.01	-5.29
Rana iberica	28.22	19.42	10.88	32.13	21.25	13.92	-1.28
Scaphiopus couchi	35.02	4.68	14.01	39.16	25.15	15.42	-2.88
Spea multiplicata	33.70	10.61	20.16	39.29	19.13	18.70	-0.60

¥ T_{opt}, optimal temperature (°C); z_{max}, maximum performance (SVL units); B_{80}, thermal performance breadth (°C); B_{80max}, upper limit of thermal performance breadth (°C); B_{80min}, lower limit of thermal performance breadth (°C); TSM, thermal safety margins calculated using average environmental temperature (°C); TSM_{tmax}, thermal safety margins calculated using maximum environmental temperature (°C).

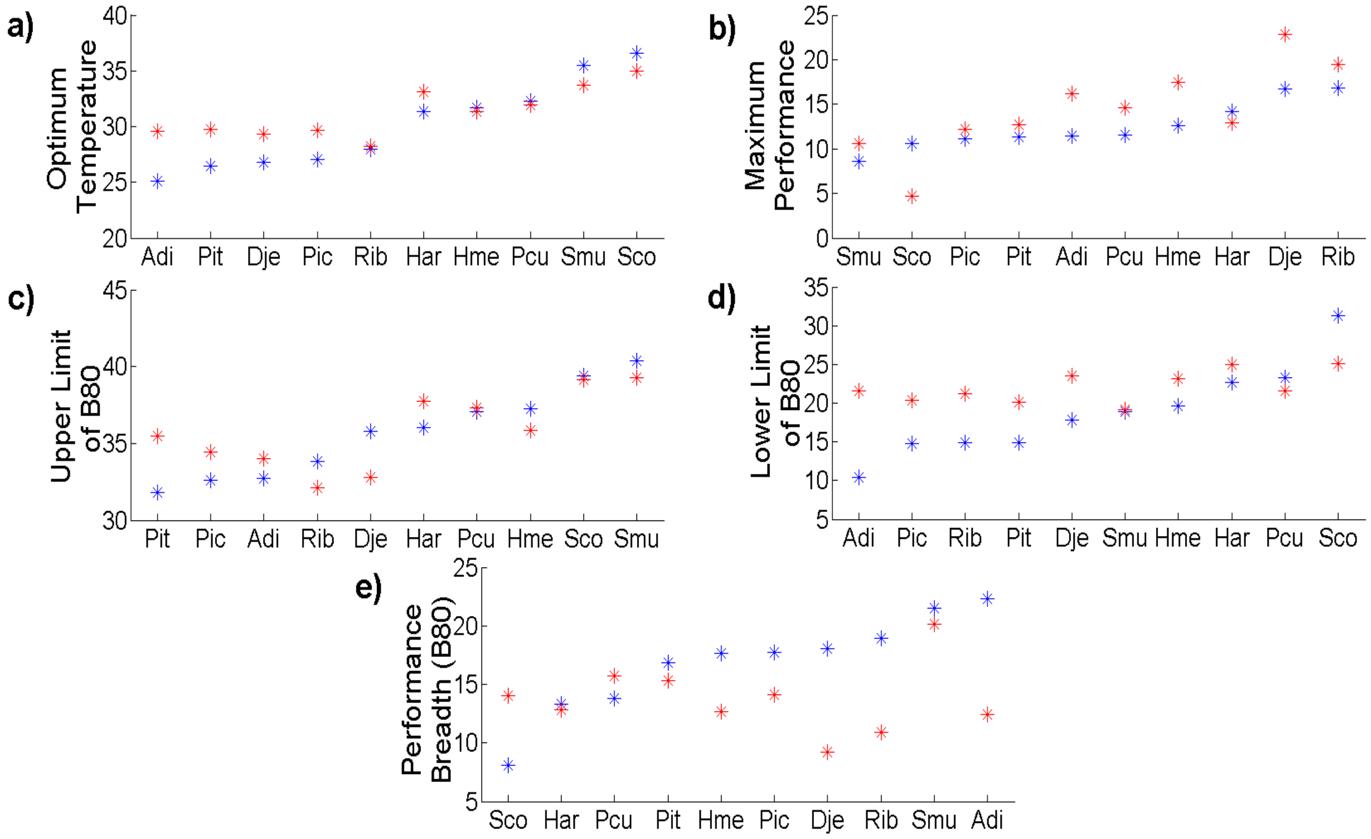
When considering habitat characteristics, optimum temperature was related to average environmental temperature (T_{avg}; Pearson's r=0.65, p=0.032; PGLS, λ = 0.88, β = 0.67 ± 0.20, t = 3.38, p = 0.008). Performance breadth increased with annual diel temperature fluctuation (DTF_{annual}; Pearson's r=0.70, p=0.016; PGLS, λ = 0.0, β = 0.62 ± 0.21, t = 2.96, p = 0.016) and decreased with minimum environmental temperature (T_{min}; PGLS, λ = 0.63, β = -0.43 ± 0.18, t = -2.37, *p* = 0.042). Upper limit of performance breadth was related to average environmental temperature (T_{avg}; PGLS, λ = 1.0, β = 0.64 ± 0.08, t = 7.59, *p* < 0.001), minimum environmental temperature (T_{min}; PGLS, λ = 0.99, β = 0.35 ± 0.11, t = 3.13, *p* = 0.012) and altitude (PGLS, λ = 1.0, β = -0.003 ± 0.001, t = -4.89, *p* = 0.001), while the lower limit of performance breadth was also related to average environmental temperature (T_{avg}; PGLS, λ = 0.81, β = 0.99 ± 0.21, t = 4.65, *p* = 0.001), minimum environmental temperature (T_{min}; PGLS, λ = 0.80, β = 0.57 ± 0.13, t = 4.39, *p* = 0.002) and altitude (Pearson’s r=-0.62, *p*=0.041; PGLS, λ = 0.0, β = -0.005 ± 0.002, t = -2.86, *p* = 0.019). We did not find any relation between maximum performance and the environmental measurements taken. In addition, all thermal physiology traits, except for performance breadth B_{80} and B_{80min}, presented significant high values of Pagel's λ (Table 4.2).

Table 4.2. Phylogenetic signal of thermal physiology traits estimated using Pagel's λ, including optimum temperature (T_{opt}), maximum performance (z_{max}), performance breadth (B_{80}) and its upper (B_{80max}) and lower limits (B_{80min}). Significant results are marked in bold.

	Pagel's λ	***p*-value**
T_{opt}	**1.00**	**0.009**
z_{max}	**1.00**	**0.038**
B_{80}	0.67	0.280
B_{80max}	**0.92**	**0.047**
B_{80min}	0.00	1.000

Comparison between juvenile and tadpole life-stages

Figure 4.1. Comparison of thermal physiology traits between life-stages of the studied amphibian species. Trait values for tadpole stage are shown in blue and for juvenile stage in red. Species codes: Adi, *Alytes dickhilleni*; Amu, *Alytes muletensis*; Dje, *Discoglossus jeanneae*; Har, *Hyla arborea*; Hme, *Hyla meridionalis*; Pcu, *Pelobates cultripes*; PiC, *Pelodytes ibericus* (Cabra population); PiT, *Pelodytes ibericus* (Toba population); Rib, *Rana iberica*; Sco, *Scaphiopus couchi*; Smu, *Spea multiplicata*.



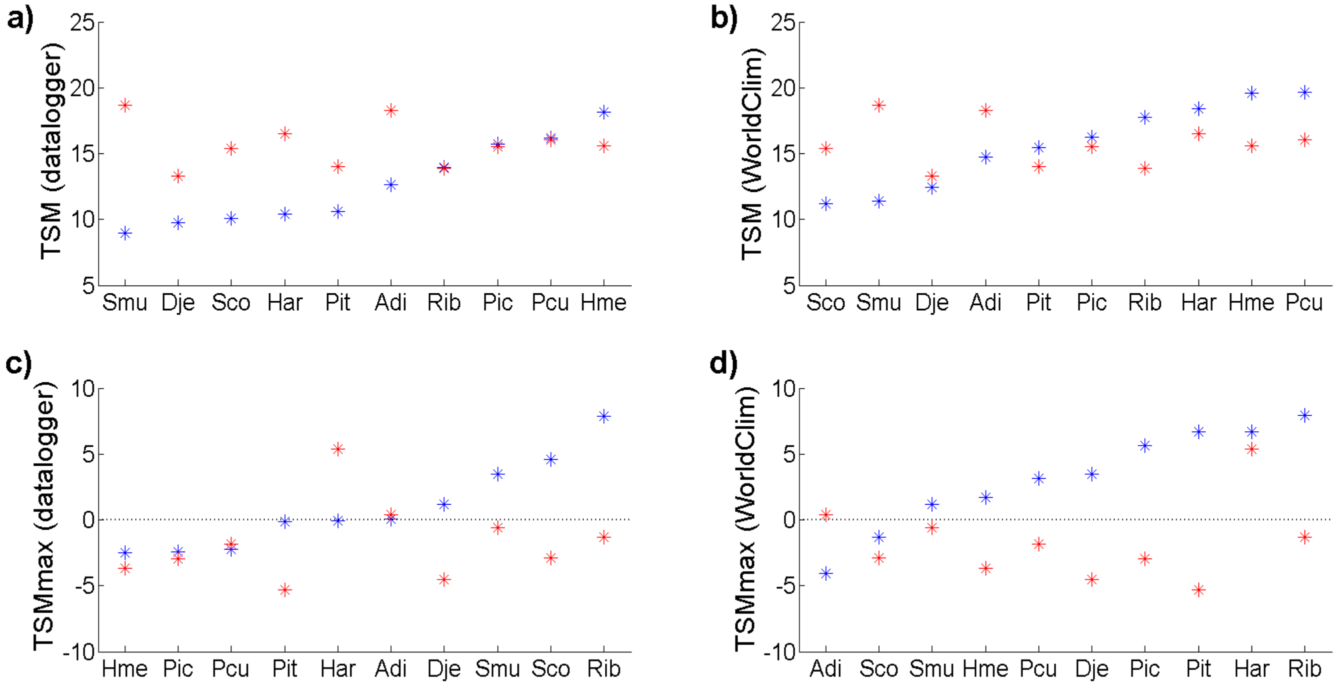
Species with lower optimum temperature in tadpole stage also had lower optimum temperature in juvenile stage (Pearson's r=0.92, p<0.001; PGLS, λ = 0.0, β = 0.51 ± 0.08, t = 6.55, p < 0.001; Figure 4.1a) and overall optimum temperature did not differ between jumping and swimming performance ($t_{(9)}$=1.61, p=0.14). However, when considering each species separately, *H. arborea* ($t_{(54)}$=5.03, p<0.001), *A. dickhilleni* ($t_{(67)}$=10.38, p<0.001) and both populations of *P. ibericus* (Toba, $t_{(48)}$=6.82, p=0.14; Cabra, $t_{(51)}$=6.53, p<0.001) had significantly higher optimum temperature on juvenile stage than on tadpole stage, while *S. multiplicata* ($t_{(39)}$=3.03, p=0.002) and *S. couchii* ($t_{(34)}$=5.04, p<0.001) had lower optimum temperature on juvenile stage.

Although swimming and jumping are two different locomotion strategies, species which had higher maximum performance on one stage also had higher performance on the other (Pearson's r=0.76, p=0.012; PGLS, λ = 0.0, β = 1.43 ± 0.44, t = 3.26, p = 0.011; Figure 4.1b).

Regarding performance breadth, there was no significant difference in its upper (B_{80max}; $t_{(9)}$=0.21, p=0.838) and lower (B_{80min}; $z_{(9)}$=1.78, p=0.075) limits between life-stages. Upper limit of performance breadth in jumping was related to that of swimming performance (Pearson's r=0.74, p=0.016; PGLS, λ = 0.26, β = 0.61 ± 0.21, t = 2.93, p = 0.019; Figure 4.1c). However, as B_{80min} is less variable in jumping than in swimming performance (Levene's test $F_{(1,18)}$= 4.56, p=0.047), there is only a relation between jumping and swimming in the lower limit of performance breadth when accounting for the phylogeny (Spearman's rho=0.50, p=0.143; PGLS, λ = 0.99, β = 0.37 ± 0.08, t = 4.89, p = 0.001; Figure 4.1d). As a result, although overall performance breadth also did not differ between life-stages (B_{80}; $t_{(9)}$=1.96, p=0.082), it is not related between jumping and swimming performance (Pearson's r=0.05, p=0.892; PGLS, λ = 0.91, β = 0.25 ± 0.16, t = 1.56, p = 0.156; Figure 4.1e).

Thermal safety margins of the juvenile stage did not differ from those of the tadpole stage (Figure 2), either using tadpole TSM estimates calculated with datalogger ($z_{(9)}$=1.78, p=0.075) or WorldClim data ($z_{(9)}$= 0.15, p=0.879), and they were also not related (datalogger, Spearman's rho=-0.01, p=0.999, PGLS, λ = 0.0, β = -0.04 ± 0.20, t = -0.21, p = 0.842; Worldclim, Spearman's rho=0.09, p=0.811, PGLS, λ = 0.0, β = -0.07 ± 0.19, t = -0.37, p = 0.724).

Figure 4.2. Comparison of Thermal Safety Margins between life-stages of the studied amphibian species. Trait values for tadpole stage are shown in blue and for juvenile stage in red. Species codes: Adi, *Alytes dickhilleni*; Amu, *Alytes muletensis*; Dje, *Discoglossus jeanneae*; Har, *Hyla arborea*; Hme, *Hyla meridionalis*; Pcu, *Pelobates cultripes*; PiC, *Pelodytes ibericus* (Cabra population); PiT, *Pelodytes ibericus* (Toba population); Rib, *Rana iberica*; Sco, *Scaphiopus couchi*; Smu, *Spea multiplicata*.



When estimated using maximum environmental temperature, thermal safety margins for the juvenile stage were mainly negative (Table 4.1, Figure 4.2), which indicate that most species experience environmental temperatures higher than their optimum temperature. Furthermore, these estimates differed from those determined using datalogger WorldClim data ($t_{(9)}$=3.15, *p*=0.012) for the tadpole stage, but not from those calculated from datalogger information ($t_{(9)}$=1.94, *p*=0.084). However, in both cases, they were not related (datalogger, Pearson's r=0.07, *p*=0.839, PGLS, λ = 0.0, β = 0.07 ± 0.32, t = 0.21, *p* = 0.839; WorldClim, Pearson's r=0.02, *p*=0.967, PGLS, λ = 0.0, β = 0.01 ± 0.28, t = 0.04, *p* = 0.867).

Both estimates of thermal safety margins (TSM and TSM_{max}) for jumping performance were related (Pearson's r=0.61, *p*=0.045; PGLS, λ = 0. 0, β = 0.30 ± 0.13, t = 2.33, *p* = 0.045). TSM was also related to performance breath (B_{80}; PGLS, λ = 0.70, β = 0.41 ± 0.18, t = 2.35, *p* = 0.043) while TSM_{max} was related to maximum environmental temperature (T_{max}; Pearson's r=-0.80, *p*=0.003, PGLS, λ = 0.0, β = -0.86 ± 0.21, t = -4.05, *p* = 0.003).

Discussion

Our results in jumping performance of juvenile amphibians show some phylogenetic inertia, with closer taxa sharing similar optimum temperature, but they also indicate that species have adapted to their thermal environment, in particular to the average environmental temperature. Although other works with ectothermic vertebrates demonstrate that "hotter is better" for locomotion performance (Van Berkum 1986, Bauwens et al. 1995, Wilson 2001), we did not find support for this hypothesis in the species analyzed. Moreover, we did observe the opposite, with species with higher optimum temperatures having lower jumping performance than populations with lower optimum temperatures (see Huey and Kingsolver 1989, Savage et al. 2004).

Juveniles with lower maximum performance had broader performance breadth, revealing a generalist-specialist trade-off and thus supporting the "Jack- of-all- temperatures-is-a-master-of-none" hypothesis. However, our results in tadpole swimming performance did not reveal such trade-off across species (Chapter 1) or at the population level (Chapter 2). Selection experiments have also provided mixed support for the importance of specialist-generalist trade-offs (Angilletta 2009), where selection for greater performance at one temperature does not always cause correlated decrease in performance at other temperatures (Bennett and Lenski 1993, Carrière and Boivin 2001, Anderson et al. 2005). As performance breadth was related to annual diel temperature fluctuation and as our analysis did not reveal phylogenetic inertia on the relation between the three traits from the performance curve (T_{opt}, z_{max} and B_{80}), it appears that adaptation to higher environmental variation (broader performance breadth) and higher average environmental temperature (increased optimum temperature) results in a loss of maximum performance.

When comparing life-stages, species with higher optimum temperature at tadpole stage also had higher optimum temperature in juvenile stage. Species such as *A. dickhilleni*, *P. ibericus* and *D. jeanneae* usually breed in late winter/early spring (Gasc et al. 1997, Ferrand et al. 2001, García-París et al. 2004) and are therefore exposed to lower environmental temperatures, resulting in lower optimum temperatures. However, when transitioning to the juvenile stage, their optimum temperature increases (although in *D. jeanneae* the increase is not statistical significant). In *R. iberica* there is no difference between stages, albeit being one of the species with lower optimum temperature in the tadpole stage. This species is associated with mountain habitats and has strong aquatic habits (Ferrand et al. 2001), which might explain the lower optimum temperature in the juvenile stage. Furthermore, some of the species that experience higher environmental temperatures during the tadpole stage, and therefore have higher optimum temperature, decrease their optimum temperature in the juvenile stage. This is the case of *S. multiplicata* and *S. couchii*, which usually breed during late spring and summer (Degenhardt et al. 1996).

Regarding locomotor performance in amphibians, previous within species studies have demonstrated decoupling between the tadpole and juvenile (froglet) stages, in particular when using size-corrected measurements of performance (Shaffer et al. 1991, Watkins 2001, Johansson et al. 2010, Brodin et al. 2013). However, we observed that species with tadpoles that swim relatively faster also have froglets which can jump relatively farther. Therefore, maximum performance may not be decoupled across life-stages in a between-species scenario, even when considering that it involves size-corrected measurements of two different locomotor strategies. Furthermore, although maximum performance in a species can be decoupled between life-stages, growth and developmental conditions at an early stage can still affect post-metamorphic traits. Reduction in development time can produce smaller juveniles, with proportionately shorter limbs, and increased oxidative stress (Ficetola and de Bernardi 2006, Gomez-Mestre et al. 2013), resulting in poor jumping performance (Tejedo et al. 2000, 2010, Ficetola and de Bernardi 2006).

Both limits of performance breadth did not differ significantly between stages, although there is a tendency for tadpoles to have lower B_{80min}. This tendency may represent an adaptive response to environmental temperatures since species with lower limits of performance breadth in the tadpole stage are also those that breed in late winter and early spring. Furthermore, juveniles are generally more specialist (narrower performance breadth) than their tadpole counterparts (broader performance breadths) and the lack of correlation in performance breadth between life-stages indicates that there is some level of decoupling in this trait.

Species with broader performance breadths also have broader thermal safety margins and there was no difference between life-stages in overall average thermal safety margin, although when using TSM estimates calculated with datalogger information there was a tendency for tadpoles to have narrower thermal safety margins than juveniles. Nevertheless, some species changed from narrower to broader thermal safety margins in the transition from tadpole to juvenile stage, while others showed the opposite. This is particularly noticeable in the comparison using tadpole TSM estimates calculated with WorldClim environmental data (Figure 4.2b). When estimating thermal safety margins using maximum environmental temperature (TSM_{max}), it revealed that the life-stages differ. Most species have negative values of TSM_{max} in their juvenile stage, which means that juveniles from almost all species already experience environmental temperatures above their optimum temperature. This is also the case for tadpoles of some species but at a much lower percentage (see also Chapter 1).Therefore, in a scenario of climate change, amphibians could be more vulnerable to increasing environmental temperatures in their juvenile terrestrial phase than during their aquatic larval stage, as exposure to temperatures above T_{opt} can cause a steep decline in performance/fitness (Huey and Stevenson 1979, Tewksbury et al. 2008) and possibly result in impaired physiological functions (Hillman et al. 2009).However, these results ought to be carefully interpreted and other aspects of the amphibians' physiology and behavior (see Navas et al. 2007) should be consider when evaluating species' vulnerability.

Apart from temperature, locomotor capacity of amphibians also depends strongly on other abiotic factors, such as water availability. Dehydration can impair jumping speed and distance, and may ultimately affect the species' thermal performance curve (Navas et al. 2008, Titon Jr. et al. 2010, Titon Jr. and Gomes 2012). In their study on locomotor performance, Titon Jr. et al. (2010) found that a subtropical species with breeding peak during austral winter (*Rhinella icterica*) was less sensitive to dehydration at 15 °C, while another which breeds mostly in spring (*Rhinella schneideri*) showed lower sensitivity at 25 °C. They also found that a species with broader breeding period (*Rhinella ornata*) had high sensitivity to dehydration at all temperatures. In cane toads (*Rhinella marina*), dehydration's effect on performance was more evident at higher temperatures (Preest and Pough 2014). Furthermore, some species can also be far less sensitive to dehydration than others and consequently, be capable of sustaining performance through high body temperatures, as seen in the heat tolerant species *Rhinella granulosa* (Prates et al. 2013). In addition to the differences in sensibility among species, the influence of environmental factors on locomotion can be complicated by interactive effects which may result in similar levels of performance at different combinations of temperature and hydration (Walvoord 2003, Prates et al. 2013).

As amphibians can be exposed to environmental temperatures above their optimum temperature (see Chapter 1), they may also need to rely on behaviors and access habitats that provide a reprieve from extreme operative temperatures (Wu and Kam 2005, Wells 2007, Sunday et al. 2014). While tadpoles are constrained to the range of temperatures available in the breeding habitat (usually a pond or stream), juveniles can thermoregulate behaviorally by exploring different microhabitats in search of less oppressive temperatures. However, in both stages, this ability to modify behaviors to thermoregulate (e.g., timing of activities) can have associated fitness costs, such as narrower foraging windows or increased predation risk, although these abilities and their costs are likely to vary with habitat (Huey and Slatkin 1976, Kearney et al. 2009, Huey et al. 2009, Sunday et al. 2014, see also Chapter 1). Amphibians also have other strategies to cope with unfavorable environmental conditions (Wells 2007), which might influence the upper and lower limits of the thermal performance breadth. For surviving low ambient temperatures, some amphibians appear to have developed tolerance to freezing or overwinter in water (Berger 1982, Licht 1991, Sinsch 1991, Pasanen and Sorjonen 1994, Stinner et al. 1994). Others hibernate in burrows (Froom 1982, Sinsch 1989, Denton and Beebee 1993, Holenweg and Reyer 2000) or use existing insulated refuges below the frostline (Froom 1982, Kwet 1996). During dry and hot seasons, amphibians can also burrow into the mud or soil to aestivate, either forming a cocoon or increasing the osmotic concentration of body fluids to reduce evaporative water loss, which is commonly accompanied by a reduction of temperature sensitivity of the metabolism (Abe 1995) and depression of metabolic rate (Secor 2005). Anurans might also exhibit specialized secretions that contribute to reducing water loss (Wygoda 1984, Abe 1995, Lillywhite et al. 1997a, 1997b) or body cooling (Lillywhite 1971). Hence, physiological adjustments and behavioral strategies can help amphibians in coping with environmental stress and still occupy biomes were conditions are harsh. This is the case of *Bufo granulosus* (a.k.a. *Rhinella* granulosa) inhabiting the Brazilian Caatinga, a semi-arid biome. Because of their patterns of activity (diurnal), its juveniles are exposed to hot and dehydrating conditions which they can sustain by having developed high thermal tolerances, keen ability to detect and uptake water, and avoidance behaviors (Navas et al. 2007).

Conclusions

Anurans' locomotor performance and surrogate morphological traits have an important phylogenetic component (Gomes et al. 2009; see also Chapter 1). Moreover, in our study of juvenile jumping performance, some traits (optimum temperature and maximum performance) appear to show latency or not be completely decoupled from the previous life-stage while others (performance breadth) showed no relation between tadpole and juvenile stages. In addition, as optimum temperature and performance breadth were influenced by environmental temperatures, adaptation to the terrestrial environment caused trade-off between traits of the thermal performance curve and it is in agreement with the perception of variation in jumping ability being also associated with niche diversification and habitat use (Zug 1978, Gomes et al. 2009).

The risk posed by juveniles already experiencing environmental temperatures above their optimum temperature, such as steep decline in fitness or impaired physiological functions (Huey and Stevenson 1979, Tewksbury et al. 2008, Hillman et al. 2009), requires a carefully interpretation. Considering that this situation is experienced by several groups of ectotherms (Sunday et al. 2014), a too simplistic approach might miss the full picture. As predictions by the IPCC (2007) also include changes in the precipitation patterns, and therefore water availability, other factors such as water balance (Titon Jr. et al. 2010, Titon Jr. and Gomes 2012) and their interactions with temperature should also be considered, in particular for species with terrestrial habits. Hence, it is important not only to determine if species are exposed to unfavorable environmental conditions but also if they have the ability to respond by changing behaviors and explore microclimates to cope with those conditions (Wu and Kam 2005, Sunday et al. 2014). Therefore, future predictions of amphibian vulnerability to climate change should incorporate information on behavioral thermoregulation as well as estimates of the energetic consequences of extended thermal retreats (Sinervo et al. 2010, Huey et al. 2012, Sunday et al. 2014).

GENERAL CONCLUSIONS

1. Previous works predicted that species from tropical and subtropical warm communities should be more vulnerable to increasing environmental temperatures. Using a physiology-based approach to determine species' Warming Tolerance and Thermal Safety Margins, the results expressed in this thesis are in agreement with previous findings.

2. Although estimates of Warming Tolerance and Thermal Safety Margins in tadpoles were mainly positive, when estimating Thermal Safety Margins using maximum environmental temperature (instead of average environmental temperature) the data also shows that several species already experience temperatures very close or even above their optimal performance temperatures. As some of the species included in this group are also from higher latitudes, this indicates that amphibians from all latitudes should develop (or have developed) strategies that provide a reprieve from extreme operative temperatures.

3. In the interspecific approach, the presence of a phylogenetic signal indicates that species show little variation within closely related taxa and is consistent with previous works on thermal physiology. Their thermal characteristics could have resulted from evolutionary phylogenetic constraints (phylogenetic inertia) and, to some extent, be evolutionarily conservative. In addition, most of the physiology traits measured, such as optimum temperature, CT_{max} and the upper and lower limits of B_{80}, were strongly related to environmental temperature, also indicating some thermal adaptation in spite of the phylogenetic inertia.

4. The two *Pelodytes* species studied (*Pelodytes ibericus* and *P. puntactus*) shared similar thermal physiology characteristics. As these species are closely related, phylogenetic inertia could have played a role in making the thermal physiology of these species (populations) evolutionarily conservative and the small variation in thermal physiology could have been achieved, or at least maintained, by genetic drift.

5. As the populations of *Pelodytes* spp. have relatively high Warming Tolerance and broad Thermal Safety Margins, the current thermal environment may not be imposing a strong selection on their thermal physiology and, thus we found no evidence for local temperature adaption of populations. This indicates that the thermal physiology of *Pelodytes* spp. populations could be reflecting environmental sorting of lineages rather than local adaptation.

6. Although the use of large databases, such as WorldClim, facilitates acquisition of environmental temperature information and gives a similar qualitative perspective to that obtained with dataloggers placed in the microhabitat, they can differ in the magnitude of the temperature estimates. Future research should consider these differences when interpreting their results and it is recommend the use of dataloggers (whenever possible) to better study the species' microhabitat. This would corroborate or complement the assessment of species’ vulnerability to increasing environmental temperatures, in particular using WT and TSM metrics.

7. In addition to playing a pervasive role in nature, predators can also induce morphological and behavioural changes in their prey. Before this thesis, it was previously unknown that predators (dragonfly larvae) could also affect the thermal physiology of their prey (tadpoles), by inducing an increase in the prey's critical thermal maximum (CT_{max}) and optimum temperature and promote shape changes in their thermal performance curve.

8. Although the herbicide Roundup did not affect the tadpoles' critical thermal maximum (CT_{max}) and optimum temperature, it did produce changes in their thermal performance curve. These were mainly the result of morphology changes in the tadpoles that resembled those induced by the presence of predator cues.

9. In the tadpole stage, between-species analysis, including the work on genus *Pelodytes*, revealed a trade-off between optimum temperature and performance breadth, where species with higher optimum temperature showed narrower performance breadths, and thus supporting "hotter is narrower". However, in a within-species level, no support for this hypothesis was found. In addition, results in juvenile jumping performance indicate that "hotter is broader", where species with higher optimum temperatures are also likely to have broader performance breadths.

10. The “Jack-of-all-temperatures-is-a-master-of-none” hypothesis predicts a generalist-specialist trade-off where selection for greater performance at one temperature causes correlated decrease in performance at other temperatures. In the studies on tadpole performance included in this thesis, none showed empirical support for this hypothesis, so that in this life-stage a “Jack-of-all-temperatures” does not have to be a master of none. However, the work on juvenile performance revealed a generalist-specialist trade-off and thus supported the “Jack-of-all-temperatures-is-a-master-of-none" hypothesis.

11. The “hotter is better” hypothesis predicts that taxa with higher optimum temperatures also have better performance at the optimum temperature than populations with lower optimum temperatures. In the studies included in this thesis, only the populations of *P. puntactus* showed that “hotter was better”. All other works in amphibian performance, whether comparing between- or within-species variation or different life-stages, did not find support for this hypothesis. Moreover, in the study of juvenile jumping performance the opposite was observed, with species with higher optimum temperatures having lower jumping performance than species with lower optimum temperatures (hotter is worse).

12. The study of juvenile jumping performance revealed that some traits (such as optimum temperature and maximum performance) also appear to show latency or not be completely decoupled from the previous life-stage while others (such as performance breadth) showed no relation between tadpole and juvenile stages.

13. In juveniles, as optimum temperature and performance breadth were influenced by environmental temperatures, adaptation to the terrestrial environment caused a trade-off between traits of the thermal performance curve and is in agreement with the perception of variation in jumping ability also being associated with niche diversification and habitat use.

14. Most amphibian juveniles may already experience environmental temperatures above their optimum temperature and appear to be more vulnerable in this stage than in the tadpole stage. However, considering that several groups of ectotherms also experience similar conditions, a too simplistic approach might miss the full picture and therefore these results should be carefully interpreted.

Final considerations

With some of the hypotheses tested in this thesis yielding mixed results in several works, further research is needed to better understand the evolution and plasticity of thermal physiology and its constraints. Recent works suggest that climate warming can lead to physiological shifts in the TPCs, indicating that thermal adaptation can occur rapidly in response to changing thermal conditions. It is also important to identify the indirect effects of physiological responses (in particular thermal physiology) on species interactions, such as predation, competition and disease transmission. Understanding the plasticity of thermal performance curves and thermal limits (CT_{max} and CT_{min}) and how these parameters are altered by environmental stressors may be critical to understanding how physiological variation can influence a species' response to climate change. Furthermore, it is important not only to determine if species are exposed to unfavorable environmental conditions but also if they have the ability to respond by changing behaviors and explore microclimates or if they possess other physiological/evolutionary mechanisms which would allow them to adapt to new conditions. This knowledge could improve physiologically based species distribution models allowing for better predictions on the impacts of climate change on biodiversity.

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Coming to the end of this thesis, I am going to end it much like I started it, with a couple of quotes from my favourite astrophysicist, and my final thoughts. When ask about improving science education, Neil deGrasse Tyson gave the following answer:

"How do we change the way science is taught? Ask anybody how many teachers truly made a difference in their life, and you never come up with more than the fingers on one hand. You remember their names, you remember what they did, you remember how they moved in front of the classroom. You know why you remember them? Because they were passionate about the subject. You remember them because they lit a flame within you. They got you excited about a subject you didn't previously care about, because they were excited about it themselves. That's what turns people on to careers in science and engineering and mathematics. That's what we need to promote."

— Neil deGrasse Tyson, in *Space Chronicles: Facing the Ultimate Frontier*

Although my PhD has been quite a harsh life experience, I was very fortunate to find amazing people and great friends when I most needed it. People like Rick and Ivan filled the role of mentors and kept my motivation high by lighting a fire within me and renewing my interest in science. I will certainly remember them for the rest of my life. My (extended) family and friends helped me achieve an emotional balance and gave me confidence to finish this PhD. Now, as I write the last words of this thesis, I go back to where I started - my parents and their interest and love for nature, which I proudly inherited. It was, it is and it will always be part of who I am.

"*I would teach how science works as much as I would teach what science knows. I would assert (given that essentially, everyone will learn to read) that science literacy is the most important kind of literacy they can take into the 21st century. I would undervalue grades based on knowing things and find ways to reward curiosity. In the end, it's the people who are curious who change the world."*

— Neil deGrasse Tyson

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ANNEXE I

Supporting information for Chapter 1

Annexe I-A. General information on species' collection sites, including country, geographical coordinates, altitude (in meters) and Köppen-Geiger classification.

Species	Country	latitude	longitude	altitude	koppen
Alytes cisternasii	Spain	37°59'39.88"N	04°54'07.82"W	585	Csa
Alytes dickhilleni	Spain	36°56'51.81"N	02°50'57.35"W	1515	Csa
Aplastodiscus ibirapitanga	Brasil	15°25'18.00"S	39°32'28.20"W	177	Aw
Bufo americanus	USA	41°41'16.33"N	80°25'33.89"W	333	Dfb
Crossodactylus schmidti	Argentina	27°05'14.52"S	54°57'01.41"W	207	Cfa
Dendropsophus anceps	Brasil	14°37'22.26"S	39°21'16.31"W	98	Af
Dendropsophus elegans	Brasil	14°37'22.26"S	39°21'16.31"W	98	Af
Dendropsophus minutus	Brasil	15°00'25.70"S	39°00'01.30"W	19	Af
Dermatonotus muelleri	Argentina	23°45'36.33"S	62°08'06.62"W	198	BSh
Discoglossus galganoi	Portugal	37°40'07.88"N	08°15'47.98"W	150	Csa
Discoglossus jeanneae	Spain	38°16'38.50"N	02°56'47.37"W	498	Csa
Elachistocleis bicolor	Argentina	27°29'32.87"S	58°45'34.97"W	63	Cfa
Epidalea calamita	Spain	37°47'11.94"N	06°04'31.22"W	498	Csa
Hyla arborea	Portugal	38°34'33.90"N	09°08'38.96"W	62	Csa
Hyla meridionalis	Spain	37°59'39.88"N	04°54'07.82"W	585	Csa
Hyla versicolor	USA	41°41'27.96"N	80°29'57.07"W	317	Dfb
Hylomantis aspera	Brasil	14°38'50.90"S	39°13'15.50"W	106	Af
Hypsiboas curupi	Argentina	27°05'20.60"S	54°57'06.54"W	201	Cfa
Hypsiboas faber	Argentina	27°05'20.45"S	54°57'03.00"W	204	Cfa
Hypsiboas raniceps	Argentina	27°25'45.43"S	58°44'37.00"W	61	Cfa
Lepidobatrachus asper	Argentina	28°49'35.00"S	64°00'14.00"W	111	BSh
Lepidobatrachus llanensis	Argentina	25°29'01.90"S	62°56'34.50"W	240	BSh
Leptodactylus bufonius	Argentina	25°18'57.82"S	59°41'47.26"W	103	Cwa
Leptodactylus mystacinus	Argentina	25°55'06.86"S	53°52'46.75"W	425	Cfa
Leptodactylus ocellatus	Brasil	14°54'30.99"S	39°01'28.94"W	10	Af
Limnmedusa macroglossa	Argentina	27°05'20.60"S	54°57'06.54"W	201	Cfa
Pelobates cultripes	Spain	37°47'28.42"N	06°04'39.82"W	453	Csa
Pelodytes ibericus	Spain	36°51'31.85"N	06°11'17.77"W	14	Csa
Pelodytes puntactus 1	Spain	38°07'09.66"N	02°41'31.02"W	1396	Csa
Pelodytes puntactus 2	Portugal	37°06'59.57"N	08°53'34.06"W	126	Csa
Phasmahyla spectabilis	Brasil	15°25'03.90"S	39°32'44.60"W	248	Aw
Phyllomedusa burmeisteri	Brasil	14°47'40.77"S	39°10'24.97"W	30	Af
Phyllomedusa tetraploidea	Argentina	27°05'31.71"S	54°56'40.92"W	238	Cfa
Physalaemus albonotatus	Argentina	27°29'32.87"S	58°45'34.97"W	63	Cfa
Physalaemus erikae	Brasil	14°36'33.88"S	39°03'40.07"W	7	Af

Annexe I-A (continuation).

Species	Country	latitude	longitude	altitude	koppen
Pipa carvalhoi	Brasil	14°37'31.17"S	39°20'58.84"W	91	Af
Pleurodema borelli	Argentina	26°46'24.00"S	65°23'12.00"W	928	Cwa
Pseudacris crucifer	USA	41°34'08.60"N	80°27'09.03"W	371	Dfb
Pseudacris feriarum	USA	35°37'35.96"N	83°41'00.53"W	482	Cfa
Pseudacris triseriata	USA	42°27'32.85"N	84°00'55.66"W	304	Dfa
Pseudis limellum	Argentina	27°25'45.43"S	58°44'37.00"W	61	Cfa
Pseudis platensis	Argentina	27°25'45.43"S	58°44'37.00"W	61	Cfa
Rana cascadae	USA	44°28'50.31"N	121°59'39.68"W	1139	Csb
Rana catesbeiana	USA	41°41'08.60"N	80°30'48.26"W	311	Dfb
Rana clamitans	USA	41°41'16.33"N	80°25'33.89"W	333	Dfb
Rana iberica	Portugal	39°18'47.45"N	07°23'06.37"W	701	Csa
Rana luteiventris	USA	46°44'07.65"N	116°55'16.02"W	863	Cfb
Rana pipiens	USA	41°41'27.96"N	80°29'57.07"W	317	Dfb
Rana sphenocephala	USA	34°54'38.00"N	76°59'15.00"W	10	Cfa
Rana sylvatica	USA	41°41'30.09"N	80°30'02.91"W	318	Dfb
Rana temporaria	Spain	43°17'42.69"N	05°16'26.78"W	378	Cfb
Rhinella arenarum	Argentina	26°37'04.00"S	65°11'11.00"W	556	Cwa
Rhinella crucifer	Brasil	15°25'18.00"S	39°32'28.20"W	177	Aw
Rhinella jimi	Brasil	13°55'20.67"S	41°07'03.21"W	376	BSh
Rhinella ornata	Argentina	27°03'29.42"S	54°49'39.82"W	410	Cfa
Rhinella schneideri	Argentina	23°53'14.20"S	61°50'55.33"W	183	BSh
Salamandra salamandra	Spain	37°59'39.88"N	04°54'07.82"W	585	Csa
Scaphiopus couchii	USA	31°54'42.84"N	111°50'16.80"W	751	BSh
Scinax acuminatus	Argentina	27°25'45.43"S	58°44'37.00"W	61	Cfa
Scinax eurydice	Brasil	14°36'33.88"S	39°03'40.07"W	7	Af
Scinax fuscovarius	Argentina	27°05'31.71"S	54°56'40.92"W	238	Cfa
Scinax juncae	Brasil	14°37'31.17"S	39°20'58.84"W	91	Af
Scinax nasicus	Argentina	27°25'45.43"S	58°44'37.00"W	61	Cfa
Scinax strigilatus	Brasil	14°38'50.90"S	39°13'15.50"W	106	Af
Spea multiplicata	USA	32°30'00.00"N	106°41'24.00"W	1329	BSk
Sphaenorhynchus pauloalvini	Brasil	14°37'22.26"S	39°21'16.31"W	98	Af
Trachycephalus venulosus	Argentina	24°33'27.18"S	60°28'03.34"W	133	Cwa
Leptodactylus sp.	Argentina	27°25'45.43"S	58°44'37.00"W	61	Cfa
Phyllomedusa sp.	Brasil	14°37'31.17"S	39°20'58.84"W	91	Af
Physalaemus sp.	Brasil	14°37'31.17"S	39°20'58.84"W	91	Af
Scinax sp.	Brasil	15°00'25.70"S	39°00'01.30"W	19	Af

Annexe I-B. WorldClim and datalogger environmental information collected for the studied species, including maximum (T_{max}), average (T_{avg}) and minimum (T_{min}) environmental temperatures and annual (DTF_{annual}) and average (DTF_{avg}) diel temperature fluctuation.

Species	WorldClim				datalogger			
	T_{max}	T_{avg}	T_{min}	DTF_{annual}	T_{max}	T_{avg}	T_{min}	DTF_{avg}
Alytes cisternasii	23.4	11.4	2.4	12.3	24.2	10.7	1.4	4.5
Alytes dickhilleni	29.2	10.4	-1.3	11.7	25.1	12.5	2.3	2.2
Aplastodiscus ibirapitanga	29.3	23.6	17.5	7.8	24.6	23.5	22.5	0.6
Bufo americanus	27.1	14.9	1.5	11.2	25.7	19.2	9.7	3.7
Crossodactylus schmidti	31.6	22.3	12.0	12.4	25.2	22.3	17.1	1.6
Dendropsophus anceps	29.3	24.2	18.8	6.6	28.4	26.7	25	0.7
Dendropsophus elegans	29.3	24.2	18.8	6.6	28.4	26.7	25	0.7
Dendropsophus minutus	29.5	24.7	19.9	6.3	32.7	27.7	24.4	2.9
Dermatonotus muelleri	35.2	26.0	14.4	13.8	41.4	27.7	21.3	14
Discoglossus galganoi	19.1	12.1	6.8	10	27.5	15.8	10.7	5.5
Discoglossus jeanneae	23.3	14.3	6.2	11.6	25.6	17.0	10.9	8.7
Elachistocleis bicolor	33.6	24.1	13.5	11.1	34.2	24.2	18	9.8
Epidalea calamita	29.1	11.6	3.2	11.9	33.6	14.4	4.9	8.0
Hyla arborea	24.6	12.9	8.9	8.1	31.4	20.9	12.4	14.5
Hyla meridionalis	30.0	12.1	3.2	12.3	34.2	13.5	5.2	5.5
Hyla versicolor	27.1	15.0	1.6	11.2	29.5	23.0	15.4	5.6
Hylomantis aspera	29.1	24.1	18.9	6.5	23.9	22.8	21.8	0.9
Hypsiboas curupi	31.6	22.7	12.0	12.4	27.8	23.8	17.9	3.0
Hypsiboas faber	31.6	22.7	12.0	12.4	26.9	23.7	18.4	1.7
Hypsiboas raniceps	33.6	24.6	13.7	11.1	38.2	25.1	12.5	8.9
Lepidobatrachus asper	35.2	24.3	10.7	14.6	40.6	28.2	19.7	10.9
Lepidobatrachus llanensis	34.9	25.3	12.8	14.1	40.7	28.5	20.3	10.8
Leptodactylus bufonius	34.6	25.2	13.6	13.4	40.1	28.1	18.6	16.3
Leptodactylus mystacinus	30.0	21.2	10.2	13.4	34.2	25.6	18	5.4
Leptodactylus ocellatus	29.5	24.7	19.9	6.3	32.7	27.7	24.4	2.9
Limnmedusa macroglossa	31.6	22.7	12.0	12.4	27.8	23.8	17.9	3.0
Pelobates cultripes	29.1	12.6	3.2	11.9	34.5	16.1	6.7	6.8
Pelodytes ibericus	19.6	13.7	7.9	8.2	27.9	18.5	10.6	5.8
Pelodytes puntactus 1	18.4	9.2	0.9	12.1	23.9	13.1	5.6	7.8
Pelodytes puntactus 2	16.9	12.4	8.3	8.4	22.0	16.5	12.8	2.3
Phasmahyla spectabilis	29.4	23.6	17.4	7.8	23.9	22.8	21.8	0.9
Phyllomedusa burmeisteri	29.5	24.5	19.2	6.4	28.4	26.7	25.0	0.7
Phyllomedusa tetraploidea	31.1	22.1	11.4	12.6	34.2	25.6	18.0	5.4
Physalaemus albonotatus	33.6	21.8	11.2	11.1	34.2	24.2	18.0	9.8
Physalaemus erikae	29.5	24.7	19.9	6.3	27.4	26.4	25.4	0.2

Annexe I-B (continuation).

Species	WorldClim				datalogger			
	T_{max}	T_{avg}	T_{min}	DTF_{annual}	T_{max}	T_{avg}	T_{min}	DTF_{avg}
Pipa carvalhoi	29.3	24.2	18.9	6.7	28.4	26.7	25	0.7
Pleurodema borelli	29.4	22.1	13.1	11.3	38.2	24.8	18.2	8.7
Pseudacris crucifer	27.2	12.3	-3.9	11.6	26.1	16.3	5.6	4.0
Pseudacris feriarum	27.4	14.5	0.7	13.7	27.3	20.7	14.1	4.7
Pseudacris triseriata	25.4	10.5	-3.9	10.6	20.8	14.7	6.7	1.8
Pseudis limellum	33.6	24.6	13.7	11.1	38.2	25.1	12.5	8.9
Pseudis platensis	33.6	24.3	13.7	11.1	38.2	25.1	12.5	8.9
Rana cascadae	23.8	12.1	0.9	11.9	15.3	9.8	4.0	1.7
Rana catesbeiana	27.2	8.5	-9.5	11.2	31.0	24.6	10.7	1.7
Rana clamitans	27.1	8.4	-9.6	11.2	25.7	20.2	9.7	3.2
Rana iberica	20.0	10.2	4.5	9.2	20.1	14.0	10.3	4.5
Rana luteiventris	27.6	12.0	-1.8	12.6	15.3	9.8	4.0	1.7
Rana pipiens	24.9	10.2	-3.8	11.2	29.5	18.1	7.0	4.4
Rana sphenocephala	29.8	18.3	5.5	11.4	25.1	20.9	15.0	2.1
Rana sylvatica	24.7	10.1	-3.9	11.2	28.9	17.0	7.1	7.2
Rana temporaria	17.6	8.7	2.6	8.8	23.9	12.9	5.3	3.1
Rhinella arenarum	30.8	22.5	10.5	12.5	30.7	27.4	22.3	1.8
Rhinella crucifer	29.3	23.6	17.5	7.8	24.6	23.5	22.5	0.6
Rhinella jimi	30.5	25.1	19.8	10.4	34.6	26.3	22.2	3.7
Rhinella ornata	30.4	21.4	10.7	12.8	30.4	23.2	17.7	3.6
Rhinella schneideri	35.4	24.9	11.6	13.8	36.6	28.2	20.1	5.1
Salamandra salamandra	30.0	12.6	2.4	12.3	24.2	10.7	1.4	4.5
Scaphiopus couchii[¥]	37.9	25.4	8.4	17.6	32.0	26.5	21.3	-
Scinax acuminatus	33.6	24.3	13.7	11.1	38.2	25.1	12.5	8.9
Scinax eurydice	29.5	24.7	19.9	6.3	27.4	26.4	25.4	0.2
Scinax fuscovarius	31.1	21.8	11.4	12.6	34.2	25.6	18.0	5.4
Scinax juncae	29.3	24.2	18.9	6.7	24.2	23.7	23.1	0.1
Scinax nasicus	33.6	24.3	13.7	11.1	38.2	25.1	12.5	8.9
Scinax strigilatus	29.1	24.1	18.9	6.5	23.9	22.8	21.8	0.9
Spea multiplicata[¥]	34.3	24.1	14.3	18.6	32.0	26.5	21.3	-
Sphaenorhynchus pauloalvini	29.3	24.2	18.8	6.6	28.4	26.7	25	0.7
Trachycephalus venulosus	34.3	25.5	14.2	13.5	38.1	26.5	15.1	9.0
Leptodactylus sp.	33.6	24.6	13.7	11.1	38.2	25.1	12.5	8.9
Phyllomedusa sp.	29.3	24.2	18.9	6.7	24.2	23.7	23.1	0.1
Physalaemus sp.	29.3	24.2	18.9	6.7	24.2	23.7	23.1	0.1
Scinax sp.	29.5	24.7	19.9	6.3	32.7	27.7	24.4	2.9

¥ datalogger information for these species were taken from Morey and Reznick (2004).

Annexe I-C. Swimming speed ± SE (cm/s) at different temperatures, TTL ± SE (total tadpole length, in cm) and number of tadpoles tested for 71 amphibian species.

Species	N	TTL	Swimming Speed									
			10°C	15°C	20°C	24°C	28°C	32°C	35°C	38°C	40°C	42°C
Alytes cisternasii	30	46.2 ± 1.2	48.7 ± 1.2	57.9 ± 2.1	63.6 ± 2.8	75.5 ± 2.7	63.6 ± 2.5	55.0 ± 2.6	-	-	-	-
Alytes dickhilleni	28	48.6 ± 0.8	43.2 ± 1.0	51.8 ± 1.4	54.2 ± 1.9	55.2 ± 2.1	54.3 ± 1.5	46.5 ± 2.9	-	-	-	-
Aplastodiscus ibirapitanga	25	35.6 ± 1.0	-	-	63.9 ± 1.9	66.1 ± 2.2	68.9 ± 2.2	70.2 ± 2.2	69.3 ± 2.1	3.5 ± 2.1	-	-
Bufo americanus	25	22.1 ± 0.2	16.0 ± 0.6	19.9 ± 0.6	22.2 ± 0.6	23.8 ± 0.9	25.4 ± 0.8	25.8 ± 0.9	33.2 ± 1.8	31.1 ± 1.7	13.9 ± 2.2	-
Crossodactylus schmidti	33	41.8 ± 1.0	-	-	61.7 ± 1.8	64.3 ± 3.0	65.0 ± 2.4	59.7 ± 3.9	-	-	-	-
Dendropsophus anceps	25	25.8 ± 0.8	-	-	36.8 ± 1.3	45.4 ± 1.7	47.6 ± 1.9	49.5 ± 1.6	49.3 ± 1.5	35.9 ± 2.8	3.3 ± 1.6	-
Dendropsophus elegans	12	21.9 ± 0.6	-	-	46.1 ± 2.1	47.3 ± 2.4	48.3 ± 2.7	51.2 ± 2.7	56.0 ± 3.6	47.3 ± 6.2	27.0 ± 4.9	-
Dendropsophus minutus	16	23.7 ± 1.2	-	-	43.2 ± 2.4	48.6 ± 3.0	52.4 ± 3.1	52.6 ± 2.5	51.7 ± 2.2	26.1 ± 5.2	-	-
Dermatonotus muelleri	19	21.6 ± 0.6	-	-	16.9 ± 0.9	22.6 ± 1.3	18.7 ± 0.9	19.9 ± 1.3	21.2 ± 1.0	24.0 ± 1.5	11.5 ± 0.6	10.0 ± 1.0
Discoglossus galganoi	14	22.9 ± 0.5	18.6 ± 1.2	23.2 ± 1.5	27.6 ± 1.2	34.0 ± 1.6	35.7 ± 1.5	33.4 ± 1.6	-	-	-	-
Discoglossus jeanneae	8	25.0 ± 0.6	23.7 ± 1.2	29.5 ± 2.1	35.3 ± 2.4	42.6 ± 3.4	40.5 ± 3.4	39.0 ± 2.8	-	-	-	-
Elachistocleis bicolor	19	23.0 ± 0.7	-	-	22.4 ± 1.3	29.9 ± 1.6	32.5 ± 1.3	32.1 ± 1.7	32.1 ± 1.4	29.2 ± 2.1	19.7 ± 2.2	-
Epidalea calamita	25	20.3 ± 0.5	12.0 ± 0.5	12.6 ± 0.7	17.3 ± 0.6	16.9 ± 0.9	18.3 ± 0.6	19.6 ± 0.7	22.4 ± 0.7	12.9 ± 1.8	-	-
Hyla arborea	30	28.1 ± 0.7	22.1 ± 1.0	27.5 ± 1.2	29.1 ± 1.2	34.5 ± 1.6	37.4 ± 1.6	37.9 ± 1.4	37.0 ± 2.3	20.8 ± 2.9	-	-
Hyla meridionalis	30	30.8 ± 0.5	24.5 ± 0.9	29.4 ± 0.8	30.3 ± 1.2	34.6 ± 1.2	37.3 ± 1.2	38.0 ± 1.4	37.0 ± 1.2	28.0 ± 2.5	-	-
Hyla versicolor	25	35.2 ± 0.6	30.2 ± 0.8	36.8 ± 0.7	42.6 ± 1.0	48.0 ± 0.8	51.6 ± 1.0	52.1 ± 1.3	51.8 ± 1.1	46.4 ± 1.7	-	-
Hylomantis aspera	25	35.6 ± 1.0	-	-	52.0 ± 1.8	58.5 ± 2.1	58.1 ± 1.8	63.3 ± 1.6	59.1 ± 3.5	16.2 ± 3.9	-	-
Hypsiboas curupi	30	23.0 ± 0.3	-	-	38.0 ± 0.8	45.7 ± 1.2	48.9 ± 1.1	44.8 ± 1.0	40.0 ± 1.3	-	-	-
Hypsiboas faber	28	19.0 ± 0.5	-	-	27.7 ± 1.0	34.5 ± 1.5	36.8 ± 1.4	37.6 ± 1.6	36.9 ± 2.0	30.2 ± 3.7	21.9 ± 4.0	-
Hypsiboas raniceps	18	41.4 ± 1.7	-	-	41.2 ± 2.6	53.3 ± 3.0	50.0 ± 2.1	57.4 ± 3.4	60.9 ± 3.2	57.3 ± 3.3	-	-
Lepidobatrachus asper	22	50.4 ± 0.6	-	-	39.4 ± 1.7	43.5 ± 1.3	47.0 ± 1.1	47.3 ± 1.9	49.8 ± 1.4	50.0 ± 1.6	44.3 ± 1.5	-
Lepidobatrachus llanensis	20	58.9 ± 1.7	-	-	34.0 ± 1.4	40.3 ± 1.0	39.7 ± 1.9	38.9 ± 1.5	39.8 ± 1.5	42.3 ± 1.9	39.1 ± 2.2	36.2 ± 3.3
Leptodactylus mystacinus	30	21.7 ± 0.2	-	-	19.0 ± 0.6	28.5 ± 0.9	29.3 ± 0.8	31.9 ± 1.1	38.3 ± 0.6	40.5 ± 0.7	38.7 ± 0.7	27.9 ± 1.8
Leptodactylus ocellatus	14	23.3 ± 0.7	-	-	27.0 ± 0.9	29.7 ± 1.3	33.6 ± 1.2	38.0 ± 2.0	39.1 ± 2.1	23.7 ± 3.8	11.6 ± 2.4	-
Leptodactylus bufonius	29	26.5 ± 0.4	-	-	41.7 ± 1.0	46.5 ± 1.2	51.1 ± 1.4	56.1 ± 1.2	56.9 ± 1.1	58.8 ± 1.5	56.4 ± 1.2	41.2 ± 3.3
Limnmedusa macroglossa	30	24.2 ± 0.4	-	-	34.7 ± 1.1	47.4 ± 1.7	50.9 ± 1.4	55.2 ± 1.2	56.7 ± 1.5	55.6 ± 1.8	31.9 ± 3.7	-

Annexe I-C (continuation).

Species	N	TTL	Swimming Speed									
			10°C	15°C	20°C	24°C	28°C	32°C	35°C	38°C	40°C	42°C
Pelobates cultripes	35	44.8 ± 1.4	32.2 ± 0.8	37.9 ± 1.4	35.2 ± 1.1	45.5 ± 1.5	46.0 ± 1.8	48.5 ± 2.2	50.5 ± 1.5	36.1 ± 3.0	-	-
Pelodytes ibericus	25	30.1 ± 0.5	18.0 ± 0.6	20.7 ± 0.5	23.3 ± 0.7	25.8 ± 0.8	24.6 ± 0.7	20.2 ± 1.0	-	-	-	-
Pelodytes puntactus 1	30	26.4 ± 0.4	16.8 ± 0.4	20.5 ± 0.6	23.9 ± 0.7	27.6 ± 0.7	26.1 ± 0.7	21.0 ± 0.7	-	-	-	-
Pelodytes puntactus 2	9	28.7 ± 1.0	17.1 ± 0.9	21.3 ± 1.1	25.2 ± 1.5	28.1 ± 1.2	26.8 ± 1.3	23.9 ± 1.8	-	-	-	-
Phasmahyla spectabilis	24	31.6 ± 0.9	-	-	56.9 ± 2.2	54.2 ± 2.0	56.7 ± 1.8	59.5 ± 1.7	49.7 ± 2.7	2.2 ± 1.0	-	-
Phyllomedusa burmeisteri	17	33.4 ± 0.6	-	-	48.5 ± 2.2	55.6 ± 2.1	54.2 ± 1.2	56.0 ± 2.6	58.3 ± 2.4	44.1 ± 4.3	26.0 ± 4.4	-
Phyllomedusa tetraploidea	21	30.2 ± 1.0	-	-	32.7 ± 1.7	42.2 ± 1.3	43.4 ± 2.4	46.0 ± 2.6	50.1 ± 3.4	47.6 ± 1.7	12.1 ± 4.5	-
Physalaemus albonotatus	18	19.5 ± 0.7	-	-	22.5 ± 0.9	30.0 ± 1.4	31.6 ± 1.4	37.8 ± 1.0	37.9 ± 1.3	35.6 ± 1.8	-	-
Physalaemus erikae	25	19.1 ± 0.4	-	-	31.8 ± 1.4	38.2 ± 1.7	40.5 ± 1.8	41.7 ± 2.2	39.5 ± 2.0	28.4 ± 1.6	6.4 ± 2.3	-
Pipa carvalhoi	21	31.3 ± 0.7	-	-	38.1 ± 1.4	40.7 ± 1.6	38.5 ± 1.3	42.0 ± 1.6	38.6 ± 2.0	21.9 ± 3.2	-	-
Pleurodema borelli	30	25.5 ± 0.4	-	-	20.7 ± 1.0	26.0 ± 1.3	25.6 ± 1.0	27.8 ± 0.8	25.1 ± 1.0	21.2 ± 1.9	9.3 ± 1.8	-
Pseudacris crucifer	25	25.6 ± 0.9	26.5 ± 1.1	32.9 ± 1.2	35.3 ± 1.2	40.5 ± 1.8	43.8 ± 1.8	41.5 ± 1.8	39.9 ± 1.6	33.7 ± 2.8	-	-
Pseudacris feriarum	15	24.2 ± 1.0	21.7 ± 1.8	26.9 ± 1.5	30.7 ± 2.3	35.0 ± 2.4	35.2 ± 2.2	34.4 ± 2.4	33.6 ± 2.0	29.1 ± 2.8	-	-
Pseudacris triseriata	23	22.3 ± 0.6	22.4 ± 0.9	27.8 ± 1.5	29.5 ± 1.3	35.1 ± 1.3	37.8 ± 1.9	32.8 ± 1.7	33.7 ± 1.6	28.7 ± 1.9	-	-
Pseudis limellum	16	23.4 ± 0.9	-	-	21.5 ± 1.0	27.6 ± 1.5	29.6 ± 2.0	31.2 ± 2.0	30.2 ± 2.1	29.3 ± 2.4	-	-
Pseudis platensis	21	47.8 ± 2.1	-	-	38.6 ± 2.5	41.7 ± 2.1	50.9 ± 3.3	51.2 ± 1.8	51.0 ± 2.7	41.2 ± 2.1	-	-
Rana cascadae	25	34.3 ± 0.6	23.3 ± 0.6	29.1 ± 0.9	26.1 ± 0.6	34.7 ± 1.3	37.9 ± 1.1	37.4 ± 0.9	37.0 ± 1.0	5.5 ± 2.1	-	-
Rana catesbeiana	25	15.6 ± 0.3	12.5 ± 0.5	20.2 ± 0.7	22.5 ± 1.1	25.4 ± 1.3	25.7 ± 1.0	27.7 ± 1.1	25.8 ± 0.9	24.6 ± 1.8	-	-
Rana clamitans	25	16.2 ± 0.3	11.9 ± 0.6	20.3 ± 0.9	20.3 ± 0.8	26.2 ± 1.4	27.9 ± 1.1	28.6 ± 1.0	27.6 ± 1.4	21.6 ± 1.8	-	-
Rana iberica	29	34.9 ± 0.6	38.1 ± 1.5	46.8 ± 1.4	52.8 ± 1.6	55.2 ± 2.2	59.2 ± 1.9	53.4 ± 2.3	-	-	-	-
Rana luteiventris	25	31.8 ± 0.6	28.9 ± 0.8	33.4 ± 1.0	34.5 ± 1.1	40.8 ± 1.1	44.8 ± 1.4	44.3 ± 1.3	42.9 ± 1.5	1.6 ± 1.0	-	-
Rana pipiens	25	23.5 ± 0.7	21.5 ± 0.8	26.9 ± 1.0	30.1 ± 1.1	38.1 ± 1.3	38.3 ± 1.5	36.3 ± 1.5	31.6 ± 2.0	22.0 ± 2.0	-	-
Rana sphenocephala	24	29.7 ± 1.1	25.2 ± 1.0	31.2 ± 1.4	31.8 ± 1.8	38.0 ± 1.8	42.7 ± 1.8	44.5 ± 2.0	43.8 ± 2.2	34.3 ± 2.1	-	-
Rana sylvatica	25	26.0 ± 0.5	19.6 ± 1.0	25.5 ± 1.3	28.4 ± 1.6	33.6 ± 2.1	36.3 ± 1.6	32.5 ± 1.9	27.5 ± 2.2	-	-	-
Rana temporaria	29	32.2 ± 0.6	22.5 ± 0.7	23.2 ± 1.0	24.0 ± 1.2	28.7 ± 1.0	28.1 ± 1.2	26.5 ± 1.5	22.5 ± 2.0	-	-	-
Rhinella arenarum	23	21.5 ± 0.6	-	-	20.0 ± 1.1	27.2 ± 1.1	29.8 ± 1.5	30.0 ± 1.2	31.1 ± 1.8	26.6 ± 2.5	15.3 ± 2.9	-
Rhinella crucifer	25	15.9 ± 0.4	-	-	17.9 ± 0.8	19.4 ± 0.7	21.2 ± 1.0	22.5 ± 1.0	21.5 ± 0.8	17.6 ± 1.5	2.4 ± 0.9	-
Rhinella jimi	24	22.3 ± 0.9	-	-	42.3 ± 2.1	47.9 ± 2.5	46.6 ± 2.3	51.3 ± 2.4	52.5 ± 2.0	45.5 ± 2.1	20.4 ± 3.8	-
Rhinella ornata	18	21.5 ± 0.6	-	-	17.2 ± 0.8	19.9 ± 0.8	21.8 ± 0.9	24.1 ± 1.3	24.8 ± 1.3	20.9 ± 1.8	2.9 ± 0.9	-
Rhinella schneideri	28	20.2 ± 0.3	-	-	18.5 ± 0.4	20.9 ± 0.6	23.8 ± 0.6	24.5 ± 0.5	27.0 ± 0.6	27.0 ± 0.9	24.4 ± 0.8	2,7 ± 0.9

Annexe I-C (continuation).

Species	N	TTL	Swimming Speed									
			10°C	15°C	20°C	24°C	28°C	32°C	35°C	38°C	40°C	42°C
Salamandra salamandra	25	42.6 ± 0.6	45.4 ± 1.0	54.4 ± 1.3	60.1 ± 1.5	68.2 ± 1.5	71.0 ± 2.1	69.9 ± 2.0¥	-	-	-	-
Scaphiopus couchii	28	19.2 ± 0.4	-	-	11.2 ± 0.5	17.3 ± 0.5	15.5 ± 0.4	16.9 ± 0.4	18.9 ± 0.6	19.9 ± 1.2	14.0 ± 1.1	-
Scinax acuminatus	29	32.9 ± 0.6	-	-	34.8 ± 1.7	48.4 ± 2.5	45.3 ± 2.0	45.0 ± 2.4	54.6 ± 2.8	56.9 ± 2.8	48.9 ± 3.3	34.9 ± 3.8
Scinax eurydice	24	20.8 ± 0.5	-	-	32.4 ± 1.3	40.0 ± 1.1	38.8 ± 1.7	39.0 ± 1.4	42.2 ± 2.2	28.1 ± 3.7	13.1 ± 3.1	-
Scinax fuscovarius	28	36.0 ± 1.0	-	-	43.4 ± 1.1	50.4 ± 1.6	50.3 ± 2.1	52.3 ± 1.4	61.8 ± 2.2	52.2 ± 2.9	26.9 ± 5.5	-
Scinax juncae	25	28.5 ± 0.6	-	-	44.6 ± 1.3	51.4 ± 1.6	50.2 ± 1.8	49.9 ± 1.9	53.2 ± 3.0	45.4 ± 2.2	16.2 ± 2.9	-
Scinax nasicus	30	32.7 ± 0.6	-	-	30.3 ± 1.6	40.3 ± 2.1	41.8 ± 2.0	43.5 ± 2.0	50.6 ± 1.9	55.9 ± 2.0	52.0 ± 2.2	32.4 ± 3.7
Scinax strigilatus	25	18.9 ± 0.4	-	-	37.9 ± 1.2	40.0 ± 1.7	39.5 ± 1.1	41.4 ± 1.1	40.7 ± 1.7	6.7 ± 2.7	-	-
Spea multiplicata	24	41.3 ± 1.5	-	-	28.9 ± 1.1	30.2 ± 1.5	31.2 ± 1.6	33.7 ± 1.3	35.0 ± 1.8	33.9 ± 1.5	29.5 ± 1.5	-
Sphaenorhynchus pauloalvini	23	27.2 ± 0.6	-	-	48.6 ± 2.3	55.8 ± 2.4	58.9 ± 2.6	59.1 ± 2.6	60.2 ± 2.7	43.4 ± 3.9	-	-
Trachycephalus venulosus	20	40.0 ± 0.6	-	-	24.9 ± 1.5	36.7 ± 1.6	42.8 ± 1.4	48.2 ± 1.0	52.3 ± 1.1	49.5 ± 1.7	-	-
Leptodactylus sp.	10	25.0 ± 1.3	-	-	24.2 ± 1.7	35.4 ± 3.6	31.5 ± 3.0	33.9 ± 3.7	38.9 ± 5.2	38.7 ± 5.0	-	-
Phyllomedusa sp.	13	32.2 ± 1.4	-	-	46.4 ± 2.5	54.4 ± 2.4	54.8 ± 2.4	56.5 ± 3.5	59.1 ± 1.7	33.7 ± 7.1	17.6 ± 6.4	-
Physalaemus sp.	15	17.9 ± 0.7	-	-	27.0 ± 1.9	34.1 ± 2.6	35.5 ± 2.5	37.9 ± 2.7	32.8 ± 2.7	13.5 ± 4.0	-	-
Scinax sp.	24	24.9 ± 0.8	-	-	42.8 ± 1.6	45.7 ± 1.6	46.1 ± 3.0	49.2 ± 2.5	46.9 ± 2.2	43.1 ± 3.1	34.9 ± 3.2	-

¥ Swimming speed at 30°C

Annexe I-D. Estimated parameters and polynomials for swimming thermal performance curves of 71 amphibians species, including height (h; log TTL/s); optimal temperature (T_{opt}, m; °C) and width (w, dimensionless). See Izem and Kingsolver (2005) for further details. Fourth degree polynomial, $ax^4+bx^3+cx^2+dx+e$.

Species	TMV method			nlinfit/nlparci			Polynomial				
	T_{opt}	w	h	m ± SE	w ± SE	h ± SE	a	b	c	d	e
Alytes cisternasii	24.08	1.77	0.00	24.08 ± 0.46	1.77 ± 0.11	0.00 ± 0.71	0.0015	-0.0092	-0.2992	0.0000	27.2702
Alytes dickhilleni	25.15	1.48	0.00	25.14 ± 0.46	1.48 ± 0.06	0.00 ± 0.37	-0.0008	-0.0104	-0.0549	0.0000	16.9205
Aplastodiscus ibirapitanga	32.75	1.27	0.00	32.75 ± 0.10	1.27 ± 0.02	0.00 ± 0.31	-0.0088	-0.1542	-0.7064	0.0000	27.0676
Bufo americanus	34.26	1.94	0.00	34.26 ± 0.17	1.94 ± 0.03	0.00 ± 0.22	-0.0075	-0.1646	-0.9863	0.0000	28.0632
Crossodactylus schmidti	28.01	1.68	0.00	28.01 ± 1.05	1.68 ± 0.05	0.00 ± 3.30	-0.0084	-0.0702	-0.2010	0.0000	26.4376
Dendropsophus anceps	33.85	1.31	0.00	33.85 ± 0.12	1.31 ± 0.02	0.00 ± 0.34	-0.0061	-0.1053	-0.4960	0.0000	26.6377
Dendropsophus elegans	34.80	1.23	0.00	34.80 ± 0.30	1.24 ± 0.06	0.02 ± 0.91	-0.0038	-0.0841	-0.5011	0.0000	31.3182
Dendropsophus minutus	32.56	1.39	0.00	32.56 ± 0.33	1.39 ± 0.07	0.00 ± 0.92	-0.0074	-0.1155	-0.5264	0.0000	32.7823
Dermatonotus muelleri	36.04	1.21	0.00	36.04 ± 0.18	1.21 ± 0.02	0.00 ± 0.22	-0.0014	-0.0269	-0.1333	0.0000	11.8865
Discoglossus galganoi	28.58	1.66	0.00	28.58 ± 0.66	1.66 ± 0.10	0.00 ± 0.66	-0.0014	-0.0411	-0.3887	0.0000	26.3417
Discoglossus jeanneae	26.76	1.52	0.00	26.76 ± 1.36	1.52 ± 0.17	0.00 ± 1.33	0.0006	0.0001	-0.1659	0.0000	25.3365
Elachistocleis bicolor	33.81	1.34	0.00	33.86 ± 0.31	1.35 ± 0.04	0.04 ± 0.36	-0.0026	-0.0350	-0.1462	0.0000	19.3649
Epidalea calamita	32.02	1.75	0.00	32.02 ± 0.26	1.75 ± 0.05	0.00 ± 0.21	-0.0022	-0.0540	-0.3760	0.0000	18.4219
Hyla arborea	31.31	1.67	0.00	31.31 ± 0.24	1.67 ± 0.05	0.00 ± 0.27	-0.0024	-0.0576	-0.4135	0.0000	23.7759
Hyla meridionalis	31.68	1.76	0.00	31.68 ± 0.26	1.76 ± 0.05	0.00 ± 0.24	-0.0017	-0.0408	-0.2916	0.0000	22.0532
Hyla versicolor	31.73	1.65	0.00	31.73 ± 0.36	1.65 ± 0.04	0.00 ± 0.28	-0.0003	-0.0115	-0.1585	0.0000	24.9450
Hylomantis aspera	32.72	1.32	0.00	32.72 ± 0.16	1.32 ± 0.03	0.00 ± 0.41	-0.0074	-0.1217	-0.5353	0.0000	24.2177
Hypsiboas curupi	27.64	1.80	0.00	27.64 ± 0.33	1.80 ± 0.09	0.00 ± 0.91	0.0105	0.0094	-0.6259	0.0000	38.3028
Hypsiboas faber	32.12	1.46	0.00	32.12 ± 0.34	1.46 ± 0.06	0.01 ± 0.62	-0.0033	-0.0335	-0.1630	0.0000	29.0738
Hypsiboas raniceps	35.84	1.31	0.00	35.86 ± 0.34	1.31 ± 0.03	0.00 ± 0.32	-0.0039	-0.0790	-0.4335	0.0000	19.6986
Lepidobatrachus asper	35.90	1.69	0.00	35.90 ± 0.37	1.69 ± 0.07	0.00 ± 0.31	-0.0025	-0.0406	-0.2034	0.0000	16.8422
Lepidobatrachus llanensis	37.71	1.19	0.00	37.71 ± 0.53	1.19 ± 0.05	0.00 ± 0.24	-0.0004	-0.0087	-0.0503	0.0000	8.4445
Leptodactylus bufonius	36.65	1.68	0.00	36.65 ± 0.18	1.68 ± 0.05	0.00 ± 0.44	-0.0065	-0.1215	-0.6879	0.0000	38.2517
Leptodactylus mystacinus	37.99	1.65	0.00	37.99 ± 0.11	1.65 ± 0.02	0.00 ± 0.18	-0.0110	-0.2090	-1.1140	0.0000	31.2360
Leptodactylus ocellatus	32.60	0.81	0.00	32.60 ± 0.52	0.81 ± 0.05	0.00 ± 0.83	-0.0001	-0.0046	-0.0718	0.0000	13.5534
Limnmedusa macroglossa	35.31	1.32	0.00	35.31 ± 0.11	1.32 ± 0.02	0.00 ± 0.32	-0.0062	-0.1181	-0.6435	0.0000	32.5454

Annexe I-D (continuation).

Species	TMV method			nlinfit/nlparci			Polynomial				
	T_{opt}	w	h	m ± SE	w ± SE	h ± SE	a	b	c	d	e
Pelobates cultripes	32.25	1.77	0.00	32.25 ± 0.24	1.77 ± 0.05	0.00 ± 0.23	-0.0023	-0.0551	-0.3798	0.0000	20.4223
Pelodytes ibericus	25.33	1.67	0.00	25.33 ± 0.37	1.67 ± 0.09	0.00 ± 0.33	-0.0003	-0.0120	-0.1357	0.0000	14.1608
Pelodytes puntactus 1	25.66	1.67	0.00	25.66 ± 0.26	1.67 ± 0.06	0.00 ± 0.25	-0.0004	-0.0173	-0.2052	0.0000	17.2620
Pelodytes puntactus 2	25.37	1.65	0.00	25.38 ± 0.60	1.66 ± 0.10	0.00 ± 0.43	0.0001	-0.0047	-0.1265	0.0000	15.9634
Phasmahyla spectabilis	31.64	1.26	0.00	31.64 ± 0.39	1.26 ± 0.06	0.01 ± 0.93	-0.0043	-0.0822	-0.4051	0.0000	24.3398
Phyllomedusa burmeisteri	33.72	1.33	0.00	33.71 ± 0.26	1.33 ± 0.05	0.00 ± 0.53	-0.0034	-0.0549	-0.2327	0.0000	22.9015
Phyllomedusa tetraploidea	35.37	1.21	0.00	35.37 ± 0.13	1.21 ± 0.02	0.00 ± 0.40	-0.0050	-0.1071	-0.6106	0.0000	21.8353
Physalaemus albonotatus	35.17	1.66	0.00	35.17 ± 0.44	1.66 ± 0.06	0.00 ± 0.56	-0.0088	-0.1417	-0.7244	0.0000	33.1708
Physalaemus erikae	32.80	1.40	0.00	32.80 ± 0.19	1.40 ± 0.03	0.00 ± 0.47	-0.0063	-0.0879	-0.3699	0.0000	30.8991
Pipa carvalhoi	32.79	1.25	0.00	32.78 ± 0.27	1.25 ± 0.05	0.00 ± 0.47	-0.0029	-0.0489	-0.2072	0.0000	16.6927
Pleurodema borelli	33.29	1.37	0.00	33.29 ± 0.19	1.37 ± 0.03	0.00 ± 0.23	-0.0027	-0.0376	-0.1431	0.0000	14.5288
Pseudacris crucifer	30.38	1.68	0.00	30.38 ± 0.38	1.68 ± 0.07	0.00 ± 0.45	-0.0009	-0.0247	-0.2382	0.0000	28.9137
Pseudacris feriarum	29.38	1.67	0.00	29.38 ± 0.72	1.67 ± 0.10	0.00 ± 0.64	-0.0002	-0.0082	-0.1389	0.0000	24.8734
Pseudacris triseriata	28.38	1.75	0.00	28.38 ± 1.05	1.67 ± 0.15	0.00 ± 1.11	0.0005	-0.0031	-0.1787	0.0000	28.2670
Pseudis limellum	32.80	1.53	0.00	32.34 ± 1.91	1.44 ± 0.20	0.03 ± 1.66	-0.0020	-0.0203	-0.1142	0.0000	20.2888
Pseudis platensis	31.96	1.71	0.00	31.96 ± 0.51	1.71 ± 0.14	0.00 ± 0.69	0.0002	-0.0188	-0.2511	0.0000	19.0622
Rana cascadae	31.21	1.30	0.00	31.21 ± 0.12	1.30 ± 0.02	0.00 ± 0.16	-0.0012	-0.0346	-0.2826	0.0000	15.9183
Rana catesbeiana	32.33	1.50	0.00	32.33 ± 0.72	1.50 ± 0.05	0.00 ± 0.48	-0.0007	-0.0145	-0.1277	0.0000	26.0103
Rana clamitans	31.92	1.59	0.00	31.92 ± 0.30	1.59 ± 0.04	0.00 ± 0.30	-0.0018	-0.0435	-0.3441	0.0000	28.8834
Rana iberica	27.95	1.65	0.00	27.95 ± 0.47	1.65 ± 0.06	0.00 ± 0.44	-0.0020	-0.0387	-0.2711	0.0000	27.6247
Rana luteiventris	30.89	1.29	0.00	30.89 ± 0.11	1.29 ± 0.02	0.00 ± 0.18	-0.0014	-0.0419	-0.3430	0.0000	20.2218
Rana pipiens	28.60	1.65	0.00	28.60 ± 0.37	1.65 ± 0.07	0.00 ± 0.44	-0.0003	-0.0189	-0.2676	0.0000	27.6574
Rana sphenocephala	32.39	1.79	0.00	32.39 ± 0.26	1.79 ± 0.05	0.00 ± 0.28	-0.0025	-0.0613	-0.4570	0.0000	27.4492
Rana sylvatica	28.72	1.70	0.00	28.72 ± 0.41	1.70 ± 0.08	0.00 ± 0.41	-0.0011	-0.0281	-0.2643	0.0000	23.1788
Rana temporaria	27.64	2.11	0.00	28.14 ± 0.69	2.28 ± 0.22	0.58 ± 0.65	-0.0019	-0.0494	-0.3382	0.0000	18.8674

Annexe I-D (continuation).

Species	TMV method			nlinfit/nlparci			Polynomial				
	T_{opt}	w	h	m ± SE	w ± SE	h ± SE	a	b	c	d	e
Rhinella arenarum	34.03	1.35	0.00	34.03 ± 0.21	1.35 ± 0.04	0.00 ± 0.35	-0.0032	-0.0493	-0.2305	0.0000	19.6915
Rhinella crucifer	34.02	1.33	0.00	34.02 ± 0.18	1.33 ± 0.03	0.00 ± 0.34	-0.0043	-0.0793	-0.4032	0.0000	19.6334
Rhinella jimi	34.86	1.26	0.00	34.86 ± 0.13	1.26 ± 0.03	0.00 ± 0.45	-0.0058	-0.1181	-0.6384	0.0000	31.1997
Rhinella ornata	34.67	1.29	0.00	34.67 ± 0.13	1.29 ± 0.03	0.00 ± 0.24	-0.0037	-0.0752	-0.4157	0.0000	15.7294
Rhinella schneideri	36.51	1.26	0.00	36.51 ± 0.07	1.26 ± 0.01	0.00 ± 0.15	-0.0031	-0.0725	-0.4571	0.0000	18.4047
Salamandra salamandra	28.03	1.68	0.00	28.03 ± 0.62	1.68 ± 0.06	0.00 ± 0.51	-0.0027	-0.0575	-0.3989	0.0000	28.1725
Scaphiopus couchii	36.61	1.18	0.00	36.61 ± 0.15	1.18 ± 0.02	0.00 ± 0.16	-0.0022	-0.0508	-0.3075	0.0000	12.4851
Scinax acuminatus	37.63	1.21	0.00	37.63 ± 0.17	1.21 ± 0.02	0.00 ± 0.30	-0.0024	-0.0594	-0.3818	0.0000	20.5838
Scinax eurydice	33.64	1.33	0.00	33.64 ± 0.20	1.33 ± 0.03	0.00 ± 0.50	-0.0053	-0.0803	-0.3128	0.0000	26.1772
Scinax fuscovarius	35.40	1.21	0.00	35.40 ± 0.13	1.21 ± 0.02	0.00 ± 0.31	-0.0036	-0.0806	-0.4724	0.0000	20.6663
Scinax juncae	34.87	1.20	0.00	34.87 ± 0.13	1.20 ± 0.02	0.00 ± 0.38	-0.0044	-0.0887	-0.4518	0.0000	23.3945
Scinax nasicus	37.78	1.33	0.00	37.78 ± 0.14	1.33 ± 0.02	0.00 ± 0.25	-0.0040	-0.0928	-0.5896	0.0000	22.8701
Scinax strigilatus	32.90	1.27	0.00	32.90 ± 0.14	1.27 ± 0.03	0.00 ± 0.49	-0.0097	-0.1670	-0.7389	0.0000	29.5869
Spea multiplicata	35.47	1.79	0.00	35.48 ± 0.41	1.79 ± 0.14	0.00 ± 0.55	-0.0028	-0.0494	-0.2537	0.0000	15.3740
Sphaenorhynchus pauloalvini	32.99	1.36	0.00	32.99 ± 0.25	1.36 ± 0.05	0.00 ± 0.61	-0.0049	-0.0744	-0.3297	0.0000	30.5929
Trachycephalus venulosus	35.75	1.53	0.00	35.75 ± 0.43	1.53 ± 0.04	0.00 ± 0.31	-0.0033	-0.0603	-0.3671	0.0000	20.0149
Leptodactylus sp.	36.21	1.20	0.00	36.21 ± 0.83	1.20 ± 0.07	0.00 ± 0.84	-0.0038	-0.0858	-0.5002	0.0000	19.5104
Phyllomedusa sp.	31.74	1.46	0.00	31.73 ± 0.57	1.47 ± 0.09	0.01 ± 0.94	-0.0036	-0.0476	-0.2366	0.0000	26.7904
Physalaemus sp.	31.60	1.45	0.00	31.60 ± 0.33	1.45 ± 0.07	0.00 ± 0.80	-0.0079	-0.0965	-0.4041	0.0000	31.8096
Scinax sp.	33.43	1.33	0.00	33.43 ± 0.73	1.33 ± 0.13	0.01 ± 1.65	-0.0014	-0.0245	-0.1362	0.0000	26.1789

Annexe I-E. Thermal physiology traits for 71 amphibian species, including optimum temperature (T_{opt}, °C), maximum performance (z_{max}, TTL/s), thermal performance breadth (B_{80}, °C), upper limit of thermal performance breadth (B_{80max}, °C), lower limit of thermal performance breadth (B_{80min}, °C), and critical thermal maximum (CT_{max}, °C).

Species	T_{opt}	z_{max}	B_{80}	B_{80max}	B_{80min}	CT_{max}
Alytes cisternasii	24.08 ± 0.46	15.40	16.30	31.48	15.17	38.2
Alytes dickhilleni	25.15 ± 0.46	11.44	22.33	32.70	10.36	37.6
Aplastodiscus ibirapitanga	32.75 ± 0.10	21.28	16.04	35.58	19.55	38.6
Bufo americanus	34.26 ± 0.17	14.44	10.28	38.22	27.94	41.0
Crossodactylus schmidti	28.01 ± 1.05	15.73	16.89	33.40	16.52	36.6
Dendropsophus anceps	33.85 ± 0.12	20.28	16.35	37.23	20.88	-
Dendropsophus elegans	34.80 ± 0.30	25.36	18.82	38.32	19.49	40.8
Dendropsophus minutus	32.56 ± 0.33	23.56	16.09	36.33	20.24	40.6
Dermatonotus muelleri	36.04 ± 0.18	9.86	19.69	39.89	20.20	43.6
Discoglossus galganoi	28.58 ± 0.66	15.89	13.31	33.80	20.50	38.4
Discoglossus jeanneae	26.76 ± 1.36	16.70	18.03	35.81	17.77	-
Elachistocleis bicolor	33.81 ± 0.31	14.41	17.43	38.61	21.18	41.7
Epidalea calamita	32.02 ± 0.26	10.52	12.61	36.63	24.01	39.8
Hyla arborea	31.31 ± 0.24	14.20	13.33	36.04	22.71	40.0
Hyla meridionalis	31.68 ± 0.26	12.57	17.61	37.24	19.63	39.8
Hyla versicolor	31.73 ± 0.36	15.08	20.16	39.60	19.44	40.3
Hylomantis aspera	32.72 ± 0.16	18.39	15.87	35.84	19.97	39.1
Hypsiboas curupi	27.64 ± 0.33	21.25	15.34	35.95	20.61	37.5
Hypsiboas faber	32.12 ± 0.34	19.85	17.12	38.06	20.94	41.0
Hypsiboas raniceps	35.84 ± 0.34	15.09	17.46	39.05	21.59	41.2
Lepidobatrachus asper	35.90 ± 0.37	9.99	20.71	41.12	20.40	42.6
Lepidobatrachus llanensis	37.71 ± 0.53	7.10	23.00	42.74	19.74	44.7
Leptodactylus bufonius	36.65 ± 0.18	22.72	16.35	41.17	24.82	43.3
Leptodactylus mystacinus	37.99 ± 0.11	18.97	8.98	41.27	32.30	42.8
Leptodactylus ocellatus	32.60 ± 0.52	16.77	11.09	36.85	25.76	41.7
Limnmedusa macroglossa	35.31 ± 0.11	24.65	15.38	38.70	23.32	39.9
Pelobates cultripes	32.25 ± 0.24	11.54	13.80	37.08	23.28	39.4
Pelodytes ibericus	25.33 ± 0.37	8.50	17.01	31.82	14.80	37.1
Pelodytes puntactus 1	25.66 ± 0.26	10.32	14.79	31.63	16.84	37.3
Pelodytes puntactus 2	25.37 ± 0.60	9.68	17.31	33.10	15.79	36.9
Phasmahyla spectabilis	31.64 ± 0.39	19.26	18.09	35.07	16.98	38.9
Phyllomedusa burmeisteri	33.72 ± 0.26	17.26	18.51	37.97	19.47	-
Phyllomedusa tetraploidea	35.37 ± 0.13	18.04	7.76	38.07	30.31	41.6
Physalaemus albonotatus	35.17 ± 0.44	20.00	13.59	39.20	25.61	41.1
Physalaemus erikae	32.80 ± 0.19	22.07	16.47	36.99	20.52	41.0

Annexe I-E (continuation).

Species	T_{opt}	z_{max}	B_{80}	B_{80max}	B_{80min}	CT_{max}
Pipa carvalhoi	32.79 ± 0.27	13.35	17.78	36.50	18.72	40.8
Pleurodema borelli	33.29 ± 0.19	10.61	17.32	37.63	20.31	41.0
Pseudacris crucifer	30.38 ± 0.38	17.17	19.16	37.19	18.03	39.3
Pseudacris feriarum	29.38 ± 0.72	14.87	21.05	38.01	16.96	39.2
Pseudacris triseriata	28.38 ± 1.05	16.18	20.90	38.15	17.25	39.2
Pseudis limellum	32.80 ± 1.91	13.25	17.73	39.18	21.45	41.9
Pseudis platensis	31.96 ± 0.51	11.13	14.58	37.93	23.35	42.3
Rana cascadae	31.21 ± 0.12	12.24	9.77	34.92	25.15	37.8
Rana catesbeiana	32.33 ± 0.72	17.40	22.56	39.68	17.12	38.7
Rana clamitans	31.92 ± 0.30	18.11	15.63	37.28	21.65	39.7
Rana iberica	27.95 ± 0.47	16.79	18.97	33.82	14.85	35.4
Rana luteiventris	30.89 ± 0.11	15.70	9.97	34.64	24.67	37.9
Rana pipiens	28.60 ± 0.37	16.73	16.00	35.19	19.19	38.0
Rana sphenocephala	32.39 ± 0.26	15.31	14.43	37.58	23.14	40.1
Rana sylvatica	28.72 ± 0.41	13.61	15.88	34.70	18.82	38.2
Rana temporaria	27.64 ± 0.69	8.94	17.51	33.70	16.19	36.5
Rhinella arenarum	34.03 ± 0.21	14.63	16.45	38.19	21.74	41.6
Rhinella crucifer	34.02 ± 0.18	14.76	16.80	37.35	20.55	41.8
Rhinella jimi	34.86 ± 0.13	24.76	17.71	38.05	20.34	42.6
Rhinella ornata	34.67 ± 0.13	12.23	8.86	37.60	28.74	40.7
Rhinella schneideri	36.51 ± 0.07	14.65	8.30	39.51	31.21	42.5
Salamandra salamandra	28.03 ± 0.62	16.79	15.69	33.18	17.49	36.6
Scaphiopus couchii	36.61 ± 0.15	10.61	8.09	39.41	31.33	42.6
Scinax acuminatus	37.63 ± 0.17	17.08	10.62	40.90	30.28	43.0
Scinax eurydice	33.64 ± 0.20	19.65	17.61	37.59	19.98	42.4
Scinax fuscovarius	35.40 ± 0.13	17.71	9.34	38.35	29.01	41.0
Scinax juncae	34.87 ± 0.13	19.43	18.06	37.97	19.91	-
Scinax nasicus	37.78 ± 0.14	17.20	8.51	40.91	32.40	42.6
Scinax strigilatus	32.90 ± 0.14	23.25	16.34	35.77	19.43	38.3
Spea multiplicata	35.47 ± 0.41	8.57	21.51	40.38	18.87	-
Sphaenorhynchus pauloalvini	32.99 ± 0.25	22.50	17.20	37.29	20.09	-
Trachycephalus venulosus	35.75 ± 0.43	13.12	12.27	39.85	27.58	41.9
Leptodactylus sp.	36.21 ± 0.83	16.27	8.22	39.00	30.78	-
Phyllomedusa sp.	31.74 ± 0.57	18.30	16.73	36.82	20.09	-
Physalaemus sp.	31.60 ± 0.33	21.96	14.81	35.82	21.01	-
Scinax sp.	33.43 ± 0.73	19.69	21.46	39.28	17.83	-

Annexe I-F. Warming Tolerance (WT, in °C) and two estimates of Thermal Safety Margins (in °C), calculated using average (TSM) and maximum (TSM_{tmax}) environmental temperatures.

Species	**datalogger**			**WorldClim**		
	WT	**TSM**	**TSM_{tmax}**	**WT**	**TSM**	**TSM_{tmax}**
Alytes cisternasii	14.04	13.38	-0.12	14.84	12.68	0.68
Alytes dickhilleni	12.52	12.65	0.05	8.42	14.75	-4.05
Aplastodiscus ibirapitanga	14.01	9.25	8.15	9.31	9.15	3.45
Bufo americanus	15.34	15.06	8.56	13.94	19.36	7.16
Crossodactylus schmidti	11.45	5.71	2.81	5.05	5.71	-3.59
Dendropsophus anceps	-	7.15	5.45	-	9.65	4.55
Dendropsophus elegans	12.40	8.10	6.40	11.50	10.60	5.50
Dendropsophus minutus	7.85	4.86	-0.14	11.05	7.86	3.06
Dermatonotus muelleri	2.16	8.34	-5.36	8.36	10.04	0.84
Discoglossus galganoi	10.93	12.78	1.08	19.33	16.48	9.48
Discoglossus jeanneae	-	9.76	1.16	-	12.46	3.46
Elachistocleis bicolor	7.46	9.61	-0.39	8.06	9.71	0.21
Epidalea calamita	6.17	17.62	-1.58	10.67	20.42	2.92
Hyla arborea	8.62	10.41	-0.09	15.42	18.41	6.71
Hyla meridionalis	5.63	18.18	-2.52	9.83	19.58	1.68
Hyla versicolor	10.75	8.73	2.23	13.15	16.73	4.63
Hylomantis aspera	15.15	9.92	8.82	9.95	8.62	3.62
Hypsiboas curupi	9.69	3.84	-0.16	5.89	4.94	-3.96
Hypsiboas faber	14.15	8.42	5.22	9.45	9.42	0.52
Hypsiboas raniceps	2.98	10.74	-2.36	7.58	11.24	2.24
Lepidobatrachus asper	1.96	7.70	-4.70	7.36	11.60	0.70
Lepidobatrachus llanensis	4.03	9.21	-2.99	9.83	12.41	2.81
Leptodactylus bufonius	3.19	8.55	-3.45	8.69	11.45	2.05
Leptodactylus mystacinus	8.61	12.39	3.79	12.81	16.79	7.99
Leptodactylus ocellatus	8.97	4.90	-0.10	12.18	7.90	3.10
Limnmedusa macroglossa	12.07	11.51	7.51	8.27	12.61	3.71
Pelobates cultripes	4.91	16.15	-2.25	10.31	19.65	3.15
Pelodytes ibericus	9.20	6.83	-2.57	17.50	11.63	5.73
Pelodytes puntactus 1	13.40	12.56	1.76	18.90	16.46	7.26
Pelodytes puntactus 2	14.90	8.87	3.37	20.00	12.97	8.47
Phasmahyla spectabilis	14.96	8.84	7.74	9.46	8.04	2.24
Phyllomedusa burmeisteri	-	7.02	5.32	-	9.22	4.22
Phyllomedusa tetraploidea	7.38	9.77	1.17	10.48	13.27	4.27
Physalaemus albonotatus	6.92	10.97	0.97	7.52	13.37	1.57
Physalaemus erikae	13.62	6.40	5.40	11.52	8.10	3.30

Annexe I-F (continuation).

Species	datalogger			WorldClim		
	WT	TSM	TSM$_{tmax}$	WT	TSM	TSM$_{tmax}$
Pipa carvalhoi	12.44	6.09	4.39	11.54	8.59	3.49
Pleurodema borelli	2.82	8.49	-4.91	11.62	11.19	3.89
Pseudacris crucifer	13.23	14.08	4.28	12.13	18.08	3.18
Pseudacris feriarum	11.89	8.68	2.08	11.79	14.88	1.98
Pseudacris triseriata	18.45	13.68	7.58	13.85	17.88	2.98
Pseudis limellum	3.68	7.70	-5.40	8.28	8.20	-0.80
Pseudis platensis	4.05	6.86	-6.24	8.65	7.66	-1.64
Rana cascadae	22.49	21.41	15.91	13.99	19.11	7.41
Rana catesbeiana	7.73	7.73	1.33	11.53	23.83	5.13
Rana clamitans	14.02	11.72	6.22	12.62	23.52	4.82
Rana iberica	15.32	13.95	7.85	15.42	17.75	7.95
Rana luteiventris	22.60	21.09	15.59	10.30	18.89	3.29
Rana pipiens	8.49	10.50	-0.90	13.09	18.40	3.70
Rana sphenocephala	14.96	11.49	7.29	10.26	14.09	2.59
Rana sylvatica	9.25	11.72	-0.18	13.45	18.62	4.02
Rana temporaria	12.65	14.74	3.74	18.95	18.94	10.04
Rhinella arenarum	10.88	6.63	3.33	10.78	11.53	3.23
Rhinella crucifer	17.20	10.52	9.42	12.50	10.42	4.72
Rhinella jimi	8.00	8.56	0.26	12.10	9.76	4.36
Rhinella ornata	10.26	11.47	4.27	10.26	13.27	4.27
Rhinella schneideri	5.88	8.31	-0.09	7.08	11.61	1.11
Salamandra salamandra	12.40	17.33	3.83	6.60	15.43	-1.97
Scaphiopus couchii	10.60	10.11	4.61	4.70	11.21	-1.29
Scinax acuminatus	4.81	12.53	-0.57	9.41	13.33	4.03
Scinax eurydice	15.05	7.24	6.24	12.95	8.94	4.14
Scinax fuscovarius	6.76	9.80	1.20	9.86	13.60	4.30
Scinax juncae	-	11.17	10.67	-	10.67	5.57
Scinax nasicus	4.39	12.68	-0.42	8.99	13.48	4.18
Scinax strigilatus	14.40	10.10	9.00	9.20	8.80	3.80
Spea multiplicata	-	8.97	3.47	-	11.37	1.17
Sphaenorhynchus pauloalvini	-	6.29	4.59	-	8.79	3.69
Trachycephalus venulosus	3.77	9.25	-2.35	7.57	10.25	1.45
Leptodactylus sp.	-	11.11	-1.99	-	11.61	2.61
Phyllomedusa sp.	-	8.04	7.54	-	7.54	2.44
Physalaemus sp.	-	7.90	7.40	-	7.40	2.30
Scinax sp.	-	5.73	0.73	-	8.73	3.93

ANNEXE II

Supporting information for Chapter 2

Annexe II-A. Matrices for P_{st}-F_{st} comparisons.

Population codes: Beas, BEA; Cabra, CBR; Colos/Bicos, CBS; Grazalema, GRZ; Jerez, JER; Nave do Barão, NDB; Rocha da Pena/Penina, PEN; São Luis, SLU; Vale da Telha, TEL; Toba, TOB; Trebujena, TRE; Vila do Bispo, VDB.

a) P_{st} values for critical thermal maximum (CT_{max}) above the diagonal and F_{st} values below the diagonal.

	BEA	CBR	CBS	GRZ	JER	NDB	PEN	SLU	TEL	TOB	TRE	VDB
BEA		0.329	0.000	0.000	0.000	0.000	0.215	0.000	0.000	0.435	0.000	0.000
CBR	0.189		0.392	0.255	0.452	0.357	0.000	0.469	0.268	0.604	0.313	0.379
CBS	0.208	0.179		0.270	0.000	0.000	0.311	0.000	0.247	0.343	0.257	0.000
GRZ	0.173	0.107	0.170		0.305	0.000	0.000	0.349	0.000	0.552	0.000	0.234
JER	0.158	0.087	0.158	0.102		0.000	0.334	0.000	0.269	0.483	0.305	0.000
NDB	0.183	0.145	0.053	0.142	0.122		0.236	0.220	0.000	0.495	0.000	0.000
PEN	0.206	0.173	0.063	0.172	0.144	0.033		0.368	0.000	0.533	0.000	0.285
SLU	0.188	0.165	0.038	0.157	0.137	0.031	0.052		0.319	0.415	0.351	0.000
TEL	0.183	0.158	0.085	0.151	0.129	0.045	0.064	0.050		0.533	0.000	0.206
TOB	0.345	0.301	0.324	0.312	0.282	0.291	0.323	0.306	0.291		0.569	0.405
TRE	0.207	0.130	0.201	0.131	0.134	0.166	0.202	0.181	0.174	0.314		0.216
VDB	0.182	0.147	0.081	0.144	0.125	0.047	0.066	0.055	0.034	0.284	0.166	

b) P_{st} values for optimum temperature (T_{opt}) above the diagonal and F_{st} values below the diagonal.

	CBR	CBS	GRZ	JER	NDB	PEN	SLU	TEL	TOB	TRE	VDB
CBR		0.000	0.000	0.000	0.000	0.000	0.156	0.000	0.000	0.203	0.000
CBS	0.179		0.000	0.000	0.000	0.000	0.200	0.178	0.000	0.234	0.000
GRZ	0.107	0.170		0.000	0.000	0.000	0.177	0.000	0.000	0.333	0.000
JER	0.087	0.158	0.102		0.000	0.203	0.264	0.230	0.000	0.000	0.192
NDB	0.145	0.053	0.142	0.122		0.173	0.262	0.225	0.000	0.000	0.175
PEN	0.173	0.063	0.172	0.144	0.033		0.000	0.000	0.205	0.311	0.000
SLU	0.165	0.038	0.157	0.137	0.031	0.052		0.000	0.274	0.347	0.000
TEL	0.158	0.085	0.151	0.129	0.045	0.064	0.050		0.244	0.308	0.000
TOB	0.301	0.324	0.312	0.282	0.291	0.323	0.306	0.291		0.000	0.201
TRE	0.130	0.201	0.131	0.134	0.166	0.202	0.181	0.174	0.314		0.295
VDB	0.147	0.081	0.144	0.125	0.047	0.066	0.055	0.034	0.284	0.166	

Annexe II-A (continuation).

c) P_{st} values for performance breadth (B_{80}) above the diagonal and for maximum performance (z_{max}) below the diagonal.

	CBR	**CBS**	**GRZ**	**JER**	**NDB**	**PEN**	**SLU**	**TEL**	**TOB**	**TRE**	**VDB**
CBR		0.206	0.000	0.000	0.190	0.234	0.354	0.291	0.188	0.000	0.298
CBS	0.000		0.000	0.148	0.000	0.000	0.197	0.000	0.000	0.178	0.000
GRZ	0.170	0.000		0.000	0.000	0.000	0.254	0.179	0.000	0.000	0.194
JER	0.000	0.000	0.140		0.000	0.175	0.315	0.248	0.000	0.000	0.255
NDB	0.368	0.334	0.307	0.368		0.000	0.218	0.000	0.000	0.156	0.000
PEN	0.290	0.260	0.159	0.272	0.178		0.220	0.000	0.000	0.210	0.136
SLU	0.000	0.000	0.176	0.000	0.382	0.295		0.000	0.213	0.316	0.000
TEL	0.200	0.000	0.000	0.183	0.271	0.000	0.212		0.000	0.249	0.000
TOB	0.000	0.000	0.245	0.000	0.422	0.342	0.000	0.283		0.162	0.000
TRE	0.454	0.422	0.418	0.462	0.000	0.285	0.468	0.429	0.509		0.263
VDB	0.234	0.197	0.000	0.217	0.224	0.000	0.242	0.000	0.298	0.344	

Annexe II-B. Distance matrices for comparison using Mantel tests.

Population codes: Beas, BEA; Cabra, CBR; Colos/Bicos, CBS; Grazalema, GRZ; Jerez, JER; Nave do Barão, NDB; Rocha da Pena/Penina, PEN; São Luis, SLU; Vale da Telha, TEL; Toba, TOB; Trebujena, TRE; Vila do Bispo, VDB.

a) Physiological distances above the diagonal and Nei's distance below the diagonal.

	CBR	**CBS**	**GRZ**	**JER**	**NDB**	**PEN**	**SLU**	**TEL**	**TOB**	**TRE**	**VDB**
CBR		1.131	0.961	0.929	2.807	1.864	2.545	2.473	1.727	3.173	2.209
CBS	1.564		0.870	0.803	2.301	1.579	1.902	1.926	0.815	3.080	1.499
GRZ	0.575	1.573		0.869	1.875	1.081	2.413	2.064	1.612	2.447	1.504
JER	0.461	1.735	0.616		2.568	1.916	2.685	2.643	1.142	2.722	2.123
NDB	1.548	0.424	1.630	1.581		1.102	2.936	2.102	2.841	2.156	1.329
PEN	1.787	0.477	2.028	1.659	0.266		2.253	1.474	2.311	2.556	0.877
SLU	1.999	0.262	1.841	1.865	0.299	0.446		1.044	2.144	4.583	1.610
TEL	2.473	0.785	2.017	2.101	0.473	0.590	0.498		2.448	3.957	0.901
TOB	0.891	1.755	1.138	0.966	2.024	2.408	2.170	2.412		3.467	2.047
TRE	0.444	1.533	0.493	0.604	1.486	2.056	1.734	2.022	0.880		3.120
VDB	1.706	0.703	1.686	1.754	0.499	0.601	0.551	0.356	1.832	1.582	

b) Environmental distances above the diagonal and geographic distances below the diagonal.

	CBR	**CBS**	**GRZ**	**JER**	**NDB**	**PEN**	**SLU**	**TEL**	**TOB**	**TRE**	**VDB**
CBR		7.261	4.637	8.838	6.653	6.730	7.606	8.154	2.013	7.610	8.528
CBS	371.1		4.629	3.833	0.756	2.275	0.561	1.097	5.801	3.132	1.464
GRZ	122.2	308.0		7.641	3.907	2.876	5.090	5.419	3.285	6.617	5.801
JER	186.7	246.7	74.5		4.194	5.780	3.342	3.326	8.038	1.275	3.207
NDB	328.3	78.3	246.9	178.9		1.741	1.187	1.631	5.210	3.369	2.028
PEN	331.1	73.5	250.8	183.1	4.9		2.706	2.855	5.185	5.026	3.198
SLU	384.9	17.0	318.3	254.9	80.7	75.9		0.671	6.226	2.760	1.004
TEL	397.4	61.5	317.5	248.5	70.8	67.2	48.0		6.795	2.982	0.397
TOB	71.6	321.6	144.5	186.2	290.9	292.9	336.8	356.2		6.786	7.183
TRE	177.7	234.0	76.7	22.6	170.4	174.3	243.4	240.9	169.8		3.016
VDB	404.1	81.8	319.1	248.2	75.8	73.3	68.6	20.6	365.8	242.5	

ANNEXE III

Supporting information for Chapter 3

Annexe III-A. Detailed information on laboratory conditions for rearing tadpoles during acclimation for the experiments.

On day 10, we brought sets of tadpoles into the laboratory to allow them to acclimate to lab conditions for 4 to 5 d before testing them for CT_{max} and T_{opt}. This acclimation period was chosen since previous research in adult amphibians revealed that 2 to 3 d are required to stabilize CT_{max} after a large change in acclimation temperature as occurs with outdoor environments (Hutchison 1961; Brattstrom 1968). While acclimating for the laboratory experiments, we needed to maintain each treatment's cues to avoid the loss of the induction (Relyea 2003). Therefore, tadpoles brought into the lab were kept in smaller plastic containers (40 x 25 x 20 cm) with approximately 10 L of water and a small subset of the leaf litter from the original pool from each set of tadpoles. We also added predator cages to all of the lab containers but only those tadpoles assigned to predator treatments had cages that contained a dragonfly nymph. These nymphs were fed ~100 mg grey treefrog tadpole biomass, every 2 d. Note that although the amount of grey treefrog biomass was smaller, the water volume of the lab containers was also much smaller thus maintaining the saturation of the plasticity response. Each container had ~15 tadpoles which were fed rabbit pellets *ad libitum*. The lab was held at a temperature of 20 °C with a 12L:12D photoperiod.

Annexe III-B. Description of method and apparatus used for measuring CT_{max}.

The testing apparatus consisted of a pool fit to a water bath with several 250-ml test containers filled with 200 ml of dechlorinated water at the acclimation temperature of 20 °C. The pool was heated at the determined rate using heating resistances attached to a temperature controller and temperature was homogenized using water pumps to create water movement in the pool. After achieving CT_{max}, we transferred tadpoles to cooler water (~20 °C) to allow recovery, after which they were weighed. We only tested tadpoles between 31-37 Gosner stages (Gosner 1960) since tadpoles over 38 Gosner stage have reduced thermal tolerances (Sherman 1980). Each individual was tested only once and each 250-ml container had only one tadpole per trial.

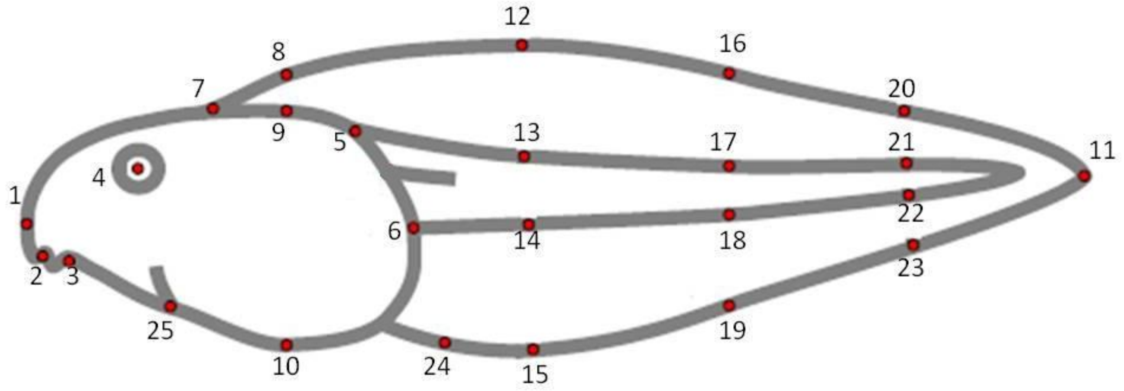
Annexe III-C. TMV method equation for calculating thermal performance curve's parameters.

The TMV method employs a polynomial function to decompose variation among TPCs into three predetermined modes of variation with biological connotation: vertical shift (faster-slower), horizontal shift (hotter-colder), and specialist-generalist trade-offs (Huey and Kingsolver, 1989; Izem and Kingsolver, 2005). Using a shape-invariant model (Eq. 1), we obtained one parameter for each direction of variation, i.e., height, optimal temperature, and width of TPC (Izem and Kingsolver 2005):

Eq. (1)

In Eq. (1), $z_i(T)$ is performance at temperature T for treatment i, z represents the common template shape of the curves, h_i is the height of TPC, $T_{opt,i}$ is the optimal temperature, and w_i is the width of TPC (also represents the specialist-generalist trade-off and is dimensionless, see Izem and Kingsolver 2005).

Annexe III-D. Description of the side-view landmarks and semi-landmarks, and linear measurements in a hypothetical tadpole.



#1 most anterior point of snout; #2 center of partially-opened mouth; #3 junction of posterior edge of lower labium and body wall; #4 center of pupil; #5 point where upper edge of tail muscle meets body; #6 point where bottom edge of tail muscle meets body; #7 point at which dorsal tail fin attaches to top of body; #8 highest point of body or tail fin 2/3rds between #1 and #6; #9 dorsal edge of body 2/3rds between #1 and #6; #10 ventral edge of body 2/3rds between #1 and #6; #11 tip of tail fin; #12 upper edge of tail fin 1/4 between #6 and #11; #13 top of tail muscle 1/4 between #6 and #11; #14 bottom of tail muscle 1/4 between #6 and #11; #15 ventral edge of tail fin 1/4 between #6 and #11; #16 upper edge of tail fin halfway between #6 and #11; #17 top of tail muscle halfway between #6 and #11; #18 bottom of tail muscle halfway between #6 and #11; #19 ventral edge of tail fin halfway between #6 and #11; #20 upper edge of tail fin 3/4 between #6 and #11; #21 top of tail muscle 3/4 between #6 and #11; #22 bottom of tail muscle 3/4 between #6 and #11; #23 ventral edge of tail fin 3/4 between #6 and #11; #24 point where center of anus meets lower edge of tail fin; #25 lower edge of body at anterior gut margin. Semilandmarks (#12 to #23) were defined by drawing a line between point #6 and #11 and perpendicular lines 25%, 50% and 75% of the distance along this line. Then, each landmark was placed at the intersections of these lines with the ventral and dorsal margins of the tail fin and tail muscle. Semilandmarks #8, #9 and #10 were generated similarly by drawing a perpendicular line 2/3 between point #1 and

#6, and the semilandmarks were placed in the intersection of the line with tail fin and dorsal/ventral edges of head/body.

Linear measurements: total tadpole length (TTL, distance between #1 and #11), body length (BL, distance between #1 and #6), body depth (BD, deepest point of the body), tail length (TL, distance between #6 and #11), muscle depth (MD, deepest point of the muscle) and tail depth (TD, maximum depth of the tail fin).

ANNEXE IV

Supporting information for Chapter 4

Annexe IV-A. Location and thermal environmental characteristics of the collection sites of the studied amphibian species, including maximum (T_{max}), average (T_{avg}) and minimum (T_{min}) environmental temperatures and annual diel temperature fluctuation (DTF_{annual}) in °C. Altitude is presented in meters (m).

Species	**Country**	**latitude**	**longitude**	**altitude**	T_{max}	T_{avg}	T_{min}	DTF_{annual}
Alytes dickhilleni	Spain	36°56'51.81"N	02°50'57.35"W	1515	29.2	11.3	-1.3	11.7
Alytes muletensis	Spain	39°51'27.20"N	02°50'14.57"E	296	27.7	15.6	5.7	7.8
Discoglossus jeanneae	Spain	38°16'38.50"N	02°56'47.37"W	498	33.8	16	3	11.6
Hyla arborea	Portugal	38°34'33.90"N	09°08'38.96"W	62	27.7	16.6	8.2	8.1
Hyla meridionalis	Spain	37°59'39.88"N	04°54'07.82"W	585	35	15.7	2.4	12.3
Pelobates cultripes	Spain	37°47'28.42"N	06°04'39.82"W	453	33.8	15.9	3.2	11.9
Pelodytes ibericus - Cabra	Spain	37°30'45.82"N	04°21'41.33"W	960	32.6	14.1	1.3	11.8
Pelodytes ibericus - Toba	Spain	37°59'39.88"N	04°54'07.82"W	585	35	15.7	2.4	12.3
Rana iberica	Portugal	39°18'47.45"N	07°23'06.37"W	701	29.5	14.3	4.5	9.2
Scaphiopus couchi	USA	31°54'42.84"N	111°50'16.80"W	751	37.9	19.6	2	17.6
Spea multiplicata	USA	32°30'00.00"N	106°41'24.00"W	1329	34.3	15	-4.6	18.6

Annexe IV-B. Snout-Vent length (SVL; in mm) and maximum jumping distance of juveniles at each test temperature for the studied amphibian species. All measurements are accompanied by ± SE.

Species[¥]	N	SVL	Jumping Performance										
			10°C	15°C	20°C	24°C	28°C	32°C	34°C	36°C	38°C	40°C	42°C
Adi	41	17.9 ± 0.3	16.2 ± 0.9	20.2 ± 0.9	22.2 ± 0.8	26.6 ± 1.2	27.1 ± 1.1	27.9 ± 1.1	27.7 ± 1.0	11.9 ± 2.7	2.8 ± 1.6	-	-
Amu	14	19.1 ± 0.4	14.1 ± 0.7	19.3 ± 1.0	21.5 ± 0.9	23.7 ± 1.0	24.6 ± 1.1	26.3 ± 1.1	26.9 ± 0.9	27.8 ± 1.6	24.5 ± 2.0	4.0 ± 2.4	-
Dje	4	8.5 ± 0.3	9.3 ± 0.4	12.1 ± 0.8	13.9 ± 0.8	16.6 ± 0.9	17.2 ± 1.2	17.9 ± 0.7	12.7 ± 5.0	0.0 ± 0.0	-	-	-
Har	26	12.5 ± 0.3	7.9 ± 0.8	10.4 ± 0.9	12.1 ± 1.0	13.5 ± 1.3	14.6 ± 1.2	15.2 ± 1.2	16.1 ± 1.4	18.2 ± 1.5	11.8 ± 1.7	9.6 ± 1.5	0.0 ± 0.0
Hme	44	13.4 ± 0.2	12.3 ± 0.5	15.2 ± 0.6	17.5 ± 0.6	20.2 ± 0.6	21.5 ± 0.8	22.4 ± 0.8	22.8 ± 0.8	18.8 ± 2.8	13.0 ± 3.1	0.2 ± 0.2	-
Pcu	15	23.8 ± 0.8	20.1 ± 1.4	24.6 ± 1.6	27.1 ± 2.0	31.4 ± 2.0	32.2 ± 2.1	33.3 ± 2.0	35.2 ± 2.0	32.1 ± 3.5	29.0 ± 3.6	12.0 ± 3.7	3.8 ± 3.8
PiC	27	12.7 ± 0.1	7.4 ± 0.4	10.5 ± 0.3	12.6 ± 0.3	14.0 ± 0.4	14.7 ± 0.5	15.8 ± 0.5	12.7 ± 1.3	-	-	-	-
PiT	24	13.4 ± 0.1	8.2 ± 0.3	11.1 ± 0.3	13.5 ± 0.3	15.5 ± 0.6	16.8 ± 0.4	16.6 ± 0.3	15.1 ± 1.2	-	-	-	-
Rib	42	13.3 ± 0.2	13.9 ± 0.7	21.2 ± 0.8	23.1 ± 0.8	24.8 ± 0.8	22.7 ± 1.2	11.1 ± 1.7	-	-	-	-	-
Sco	8	9.3 ± 0.2	-	0.5 ± 0.4	2.9 ± 0.3	3.6 ± 0.3	3.8 ± 0.4	4.0 ± 0.4	4.4 ± 0.5	4.3 ± 0.3	4.3 ± 0.3	2.8 ± 0.8	1.2 ± 0.8
Smu	17	15.0 ± 0.2	-	11.4 ± 0.6	12.9 ± 0.4	13.4 ± 0.5	15.1 ± 0.6	16.1 ± 0.7	15.2 ± 0.8	15.5 ± 0.7	14.2 ± 0.7	12.3 ± 1.1	7.2 ± 1.6

[¥] Species codes: Adi, *Alytes dickhilleni*; Amu, *Alytes muletensis*; Dje, *Discoglossus jeanneae*; Har, *Hyla arborea*; Hme, *Hyla meridionalis*; Pcu, *Pelobates cultripes*; PiC, *Pelodytes ibericus* (Cabra population); PiT, *Pelodytes ibericus* (Toba population); Rib, *Rana iberica*; Sco, *Scaphiopus couchi*; Smu, *Spea multiplicata*.

Annexe IV-C. Estimated parameters and polynomials for jumping thermal performance curves of juveniles from the studied amphibians species, including height (SVL units); optimal temperature (T_{opt}, m; °C) and width (w, dimensionless). See Izem and Kingsolver (2005) for further details. Fourth degree polynomial, $ax^4+bx^3+cx^2+dx+e$.

Species	**TMV method**			**nlinfit/nlparci**			**Polynomial**				
	T_{opt}	**w**	**h**	**m ± SE**	**w ± SE**	**h ± SE**	**a**	**b**	**c**	**d**	**e**
Alytes dickhilleni	29.57	1.38	0.00	29.77 ± 0.16	1.37 ± 0.02	0.05 ± 0.22	-0.0014	-0.0372	-0.3033	0.0000	22.3982
Alytes muletensis	33.23	1.24	0.00	33.22 ± 0.18	1.26 ± 0.02	-0.12 ± 0.23	-0.0010	-0.0323	-0.2820	0.0000	19.2444
Discoglossus jeanneae	29.29	1.31	0.00	29.29 ± 0.28	1.31 ± 0.05	0.00 ± 0.83	-0.0030	-0.0807	-0.6089	0.0000	29.8517
Hyla arborea	33.09	1.33	0.00	33.08 ± 0.26	1.34 ± 0.03	-0.03 ± 0.27	-0.0007	-0.0215	-0.1969	0.0000	17.1192
Hyla meridionalis	31.31	1.36	0.00	31.30 ± 0.16	1.37 ± 0.02	0.01 ± 0.22	-0.0012	-0.0344	-0.2974	0.0000	23.7314
Pelobates cultripes	31.95	1.39	0.00	31.95 ± 0.36	1.39 ± 0.04	0.00 ± 0.40	-0.0007	-0.0201	-0.1854	0.0000	20.2720
Pelodytes ibericus 1	29.66	1.66	0.00	29.70 ± 0.35	1.66 ± 0.04	0.02 ± 0.22	-0.0022	-0.0472	-0.3236	0.0000	20.2434
Pelodytes ibericus 2	29.71	1.64	0.00	29.71 ± 0.44	1.64 ± 0.04	0.00 ± 0.27	-0.0009	-0.0247	-0.2342	0.0000	20.7582
Rana iberica	28.22	1.61	0.00	28.22 ± 0.16	1.61 ± 0.04	0.00 ± 0.30	-0.0055	-0.1171	-0.7363	0.0000	31.2094
Scaphiopus couchi	35.02	1.18	0.00	35.02 ± 0.27	1.18 ± 0.03	0.00 ± 0.16	-0.0003	-0.0077	-0.0582	0.0000	5.5066
Spea multiplicata	33.70	1.51	0.00	33.70 ± 0.38	1.51 ± 0.06	0.00 ± 0.35	-0.0008	-0.0199	-0.1497	0.0000	16.0314





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